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14. ABSTRACT
The discovery of cyanobacteria that produce high amounts of hydrogen (H2) could lead to more efficient H2 production in engineered systems. H2 oxidizing chemolithotrophs (knallgas bacteria) utilize H2 as electron donor and oxygen as electron acceptor. The goal was to determine whether H2 exchange occurs between knallgas bacteria and cyanobacteria, and whether knallgas bacteria can be used to detect cyanobacteria that overproduce H2. Culture-dependent and -independent approaches were used to investigate interactions between knallgas bacteria and cyanobacteria. The tech- niques provided sensitive, reproducible assays for novel hydrogenases and knallgas bacteria and detected the H2 flux between cyanobacteria and knallgas bacteria. Application of the techniques to natural microbial mats has not yet yielded clear evidence of such interspecies exchange in natural ecosystems. The results suggest that H2 exchange between cyanobacteria and knallgas bacteria is possible, but the effectiveness of knallgas bacteria as indicators of H2 producing cyanobacteria may be limited because our understanding of H2 flux in microbial communities is incomplete. The potential for other bacteria and other hydrogenases to consume H2 suggests a wider net be cast to better understand H2 flux in microbial systems.

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Executive Summary

Hydrogen-oxidizing chemolithotrophs (knallgas bacteria) are taxonomically diverse and are defined functionally by the ability to utilize hydrogen as electron donor and oxygen as electron acceptor and to fix carbon dioxide. In addition to chemolithotrophic growth, most knallgas bacteria can also grow heterotrophically or mixotrophically, and they can be isolated from a wide range of ecosystems, including soil, sediment, seawater, thermophilic compost, and geothermal sites. Cyanobacterial production of hydrogen and oxygen in natural habitats, even if the processes are temporally separated, suggests the possibility of a symbiotic association between knallgas bacteria and cyanobacteria. Populations of cyanobacteria that support substantial populations of knallgas bacteria are likely to overproduce H_2 under natural conditions, and would be ideal candidates for industrial production of H_2. Such associations seem most likely in habitats such as microbial mats, symbiotic associations with plants and animals, or other areas where diffusion might be limited.

The aim of the work conducted at the Georgia Institute of Technology (Georgia Tech) and described in the first section of this report was to determine whether interspecies H_2 exchange occurs between knallgas bacteria and cyanobacteria, and, if so, whether knallgas bacteria be used as indicator organisms for the detection of cyanobacteria that overproduce hydrogen. Both culture-based and cultivation-independent approaches were used to investigate interactions between knallgas bacteria and cyanobacteria. For the culture-based approach, microbes were tested for synergistic growth when cyanobacteria and knallgas bacteria were grown in co-culture or in vessels that allowed gas exchange between axenic cultures. In addition, a reverse-transcriptase quantitative PCR method was developed to measure interspecies hydrogen exchange between the knallgas bacterium *Alcaligenes hydrogenophilus* and the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. For the cultivation-independent approach, stable isotope probing was used to detect knallgas bacteria in a variety of diverse ecosystems, particularly cyanobacteria-dominated microbial mats. The techniques developed at Georgia Tech provided sensitive and reproducible assays for detection of novel knallgas bacteria and for detection of H_2 flux between cyanobacteria and knallgas

bacteria. Application of the techniques to natural microbial mats did not yield clear evidence of such interspecies exchange in natural ecosystems.

The results suggest that although H_2 exchange between cyanobacteria and knallgas bacteria is possible, the effectiveness of knallgas bacteria as indicators of H_2-producing cyanobacteria may be limited because our understanding of H_2 flux in microbial communities is incomplete. H_2 uptake was observed for almost every sample tested, but knallgas bacteria were rarely identified as the H_2 oxidizers in complex microbial mats, indicating that other members in microbial community were consuming H_2. In addition, measuring the expression of *hupS* in Ah2625 was an effective indicator of H_2 exchange *in vitro*, but the lack of detectable *hupS* expression when Ah2625 and AMC 414 were grown together indicates more research is needed to determine if this approach can be adapted to natural systems. The lack of *hupS* expression may have been due to constitutive hydrogenase activity, or it is possible that the presence of Ah2625 inhibited H_2 production by AMC 414.

The potential for other bacteria and other hydrogenases to consume H_2 suggests a wider net be cast to better understand H_2 flux in microbial systems. One possible approach would be to design a microarray chip that contains probes for genes involved in H_2 metabolism, such as hydrogenase and nitrogenase genes, to provide a transcriptomic profile of H_2 producers and consumers in a microbial community. A similar approach has been used to investigate interspecies H_2 exchange during reductive dehalogenation, and this could facilitate discovery of bacteria that contribute to H_2 flux in cyanobacteria-dominated ecosystems. Furthermore, using an isotope array approach, it may be possible to detect cyanobacteria that express nitrogenase or hydrogenase when growing under light.

The aim of the work conducted at Colorado School of Mines (CSM) and described in the second section of this report was to describe and understand the basis for H_2 exchange between H_2-oxidizing knallgas bacteria and H_2-producing cyanobacteria in terms of the microbial composition, structure, and metabolic underpinnings in a couple of natural ecosystems, with Yellowstone National Park as the dominant site. The Yellowstone geothermal ecosystem is generally considered to be supported by sulfur metabolism. However, work by John Spear and his colleagues recently showed through a

thorough molecular phylogenetic survey, along with *in situ* aqueous H_2 determination and thermodynamic modeling, that communities instead are dependent on H_2-metabolism and may dominate this and other geothermal ecosystems. H_2 is the most abundant element in the universe and the basis of diverse microbial energy metabolisms throughout the bacterial and archaeal phylogenetic domains. This wide phylogenetic occurrence suggests that H_2 metabolism arose early in the evolution of life, perhaps even in the universal ancestor, the progenote. H_2-metabolizing microorganisms are likely involved in several geological processes, including the formation of ore bodies and the erosion of rock in all environments.

Work was divided into a series of tasks the first of which involved field work in Yellowstone National Park to examine the aqueous geochemistry (H_2 concentrations) in hot springs along with sample collection of material for microbiological analyses. Water chemistry and microbial analyses occurred in the Spear environmental microbiology laboratory at CSM. All of the work was done in collaboration with Jim Spain at Georgia Tech as the tasks moved forward, and members from both groups met both in the field and shared talks at conferences. The second task was also performed at CSM to amplify genes of interest out of the environmentally obtained samples and compare them to public databases. This task also involved the collaboration of both the student and the PI (Spear) with John Peters and Eric Boyd at Montana State University. Third, highly relevant tangential findings were pursued with collaborators Alex Sessions at Caltech along with William Berelson and Frank Corsetti at the University of Southern California (USC). With collaborator Sessions at Caltech, researchers at CSM used stable isotope probing analysis of both *in situ* natural communities as well as cultivated representatives from those communities to consider the deuterium:hydrogen ratio (D:H ratio) in extracted lipids from Yellowstone hot springs. With collaborators Berelson and Corsetti at USC; experiments were conducted to analyze the hydrogenase enzymes and microbial community of a novel cyanobacterially driven laminated, mineralized, microbial mat in the form of living stromatolites that grow along the rim of a Yellowstone hot spring.

Two field trips in 2008 were conducted to Yellowstone National Park to collect biomass samples for the proposed work under the National Park Service authorized research permit to Co-PI John R. Spear. Four field trips were conducted in 2009

including one trip with Co-Pi Spain in June, and one trip with collaborator Alex Sessions (Caltech) and graduate student (Maggie Osburn, Caltech). Samples were collected in duplicate for immediate shipment to Co-PI Jim Spain at Georgia Tech, and for laboratory work at CSM. Four field trips were completed in 2010 in support of sample acquisition for laboratory analyses; one of those trips with collaborator Spain. Preliminary sample analyses (e.g. microscopy, scanning electron microscopy, and molecular approaches—16S rRNA and functional gene analysis) were completed at CSM. Supported graduate student Chuck Pepe-Ranney extracted DNA and standardized their concentrations from 20 hot spring samples to run on a bar-coded, multiplexed, Roche/Life Sciences 454 Pyrosequencer at the University of Colorado Health Science Center in February, 2010.

The results of the work conducted at CSM along with the associated collaborative efforts have been published or submitted in seven publications. From previous work (Spear et al., 2005) we know that H_2 oxidation is a common theme in the hot springs of Yellowstone National Park. By analysis of the richly colored and densely thick microbial mats around hot springs, we showed an abundance of heterotrophic, H_2-oxidizing organisms in close association with photosynthetic cyanobacterial species and *Rosiflexus spp.* By analysis of the D:H ratio in the lipids (derived from the biomass) of these mats, we showed that photosynthetic organisms fractionate H_2 to a greater degree than heterotrophs. This finding suggests that H_2 utilization can be variable within a community, at the local level, e.g., between adjacent cells. This implies that the premise of this funded work, that knallgas bacteria may be an indicator of strong H_2 production by select phototrophs is possible, but, given the reality and complexity of dense microbial mats found in a place like Yellowstone, as indicated above, it is likely difficult to trace to individual cells. With the developing technology of nano-secondary ion mass spectrometry (NanoSIMS), it may be possible to better deduce the movement of H_2 between cells—but this will only come when the probing for H_2 becomes possible on a NanoSIMS device.

By analysis of a growing, living, stromatolite (a densely laminated rock) that lives around the edges of hot springs in Yellowstone, we were able to consider the tight associations within and between cells that allows for the 'fossilization' of microbial cell mass. Heterotrophs were shown to fix both atmospheric CO_2 and vent-derived,

subsurface CO_2 to produce new biomass with old carbon dates. This was due to the age and source of the CO_2 used in biomass construction. As the biomass also incorporates silica in its growth, a laminated 'rock' is left behind. These rocks are prevalent throughout the rock record and are indicators of early life on the planet. We conclude that CO_2 utilization and fixation into biomass is most likely carried out by a variety of organisms that are oxidizing H_2. This living stromatolite system is likely a model community with which to assess the production of H_2 by one organism, and the consumption by another. Future work should involve rate and flux experiments, further investigation of the hydrogenase enzymes involved and follow up work with D:H ratio interactions in the living community. Such a model system with tight association and interaction between a relative few kinds of organisms may be an ideal location to explore H_2 production and consumption for bioenergy application.

The ubiquity of the metabolism of hydrogen oxidation in the Tree of Life makes it prohibitively difficult to target hydrogen oxidizers (i.e. knallgas microbes) via the 16S rRNA gene. Instead, the approach was to thoroughly investigate the phylogeny of the large subunit of the NiFe hydrogenase enzyme searching for possible functional gene primer sites (going after the gene instead of the microbe). The known NiFe hydrogenases are split into 4 major groups phylogenetically. One group in particular comprises non-cyanobacterial "uptake" hydrogenase genes. Due to the remarkably high genetic diversity of the NiFe hydrogenase, it is impossible to design primers that will amplify all known NiFe's *in silico*. The diversity, however, should allow targeting the non-cyanobacterial uptake group to the exclusion of the other phylogenetic groupings that would not be associated with the knallgas metabolism. Extensive *in silico* analysis has yet to reveal an appropriate primer pair for all the aforementioned uptake genes but each of the major subgroups within the uptake-clade can be targeted by a specific primer pair. The goal is to establish a manageable set of primer combinations that encompass the entire uptake group and then investigate the diversity of such genes within our samples (above) via PCR and sequencing (culture-independent methods). This work remains on-going at the completion of this grant.

PERSONNEL SUPPORTED IN ADDITION TO PIS

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Anthony Ranchou-Peyrouse, Post Doctoral Research Associate, Georgia Tech

Graham Pumphrey, Post Doctoral Research Associate, Georgia Tech

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PUBLICATIONS

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Section 1: Chemolithotrophic hydrogen-oxidizing bacteria as indicators of hydrogen producing cyanobacteria

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INTRODUCTION

Fossil fuel reserves are limited and increasing demand compounded by negative impacts on the environment drive the search for cleaner, renewable energy carriers. Hydrogen is an appealing alternative to fossil fuels, because combustion of hydrogen produces only water and does not liberate the greenhouse gas, carbon dioxide. Hydrogen can be produced by splitting water into hydrogen and oxygen. The splitting can be accomplished chemically in photochemical fuel cells, or in photovoltaics that use solar radiation indirectly for the electrolysis reaction. A third option is the production of hydrogen by photosynthetic microorganisms that split water enzymatically (65). The low conversion efficiencies of biological systems can be offset by low energy requirements and reduced initial investment costs.

Biophotolysis can be performed by both cyanobacteria and eukaryotic algae (40, 50). During direct biophotolyis photosynthetic organisms use the energy in sunlight to catalyze the reaction: $2H_2O \rightarrow 2H_2 + O_2$. The mechanism consists of water oxidation and a light-dependent transfer of electrons via photosystems II and I to ferredoxin, which donates electrons to a hydrogenase. The hydrogenase utilizes protons as a sink for the photosynthetically generated electrons, leading to the synthesis of molecular hydrogen (36, 66). Direct biophotolysis produces no carbon dioxide and results in the simultaneous generation of hydrogen and oxygen. However, the direct photoproduction of hydrogen by cyanobacteria is a transient phenomenon, due to the rapid inactivation of the hydrogenase by the oxygen that accumulates (40, 61) and the rapid uptake of hydrogen catalyzed by the hydrogenases. Moreover, the production of hydrogen by cyanobacteria requires several minutes to a few hours of anaerobic incubation in the dark to induce the synthesis and/or activation of enzymes involved in hydrogen metabolism (85).

Indirect biophotolysis involves the photosynthetic splitting of water and concomitant conversion of carbon dioxide to reduced organic compounds (44). The organic compounds are then fermented in the dark to yield hydrogen. The strategy thus separates the production of hydrogen from the production of the inhibitory oxygen. Hydrogen accumulation, however, is inhibitory unless it is removed.

The third strategy for photobiological hydrogen production is via nitrogenase. Cyanobacteria can use the reduced carbon compounds generated during photosynthesis to

drive nitrogen fixation, which releases hydrogen as an integral part of the process (17). The nitrogenase enzyme is sensitive to oxygen, which requires that nitrogen fixation be separated temporally or spatially from photosynthesis. In many cyanobacteria, nitrogen fixation takes place in specialized heterocysts where the process is driven by reduced organic compounds produced by nearby vegetative cells. Nitrogenase also requires a considerable amount of ATP for the reduction of molecular nitrogen.

Cyanobacteria possess two functionally different types of hydrogenases: an uptake hydrogenase that catalyzes consumption of hydrogen and a bidirectional hydrogenase which has the capacity to both consume and produce hydrogen (43, 95). An uptake hydrogenase is present in all nitrogen-fixing cyanobacteria examined to date (91); however, the distribution of the enzyme in non-nitrogen fixing cyanobacteria is not clear. The uptake hydrogenase catalyzes the consumption of the hydrogen produced during the fixation of nitrogen in nitrogen-fixing cyanobacteria and seems to be upregulated along with nitrogenase (91). The consumption of hydrogen is coupled to the reduction of oxygen, leading to the formation of water (the knallgas reaction) and synthesis of ATP.

The bidirectional hydrogenase is widely, but not universally, distributed among both nitrogen-fixing and non-nitrogen-fixing cyanobacteria where it catalyzes the reversible reaction: $2H^+ + 2e^- \leftrightarrow H_2$ (16). The bidirectional hydrogenase can both take up and produce hydrogen; but its biological role is not well understood and it seems likely that it plays a role in control of ion levels inside the cyanobacteria (16, 31, 91). It is thought that cyanobacteria release hydrogen in vivo as a valve to prevent accumulation of excess electrons (8).

Cyanobacteria are ideal candidates for photobiological hydrogen production because they have simple nutritional requirements: they can grow in air, water, and mineral salts with light as the only source of energy. Most studies have been done with a few well-characterized isolates and the current focus is on metabolic engineering of the known cyanobacteria. Diversity of cyanobacteria is only beginning to be explored systematically for the discovery of novel organisms that can produce hydrogen for commercial uses. The development of methods that enable the detection and cultivation of cyanobacteria that produce high amounts of hydrogen could lead to more efficient hydrogen production in engineered systems.

It seems likely that early, primitive oxygenic phototrophs would not have had the sophisticated metabolic capabilities mentioned above for conservation of hydrogen. For example, the early cyanobacteria or their progenitors might have been much simpler. It seems reasonable to assume that they produced some hydrogen and certainly they produced oxygen, both of which would be toxic or inhibitory to their own growth. Once oxygen began to accumulate, such cyanobacteria might have developed close relationships with bacteria able to remove the oxygen and hydrogen. The bidirectional hydrogenase in modern cyanobacteria is closely related to the hydrogenase in the hydrogen-oxidizing (knallgas) bacteria (10, 81). This suggests that the genes might have been acquired by horizontal transfer at some point during evolution and supports the hypothesis of a close relationship in the past.

Knallgas bacteria are taxonomically diverse and are defined functionally by the ability to utilize hydrogen as electron donor and oxygen as electron acceptor and to fix carbon dioxide. In addition to chemolithotrophic growth, most knallgas bacteria can also grow heterotrophically or mixotrophically, and they can be isolated from a wide range of ecosystems, including soil, sediment, seawater, thermophilic compost, and geothermal sites (11). Cyanobacterial production of hydrogen and oxygen in natural habitats (42), even if the processes are temporally separated, suggests the possibility of a symbiotic association between knallgas bacteria and cyanobacteria. Populations of cyanobacteria that support substantial populations of knallgas bacteria are likely to overproduce hydrogen under natural conditions, and would be ideal candidates for industrial production of hydrogen. Such associations seem most likely in habitats such as microbial mats, symbiotic associations with plants and animals, or other areas where diffusion might be limited.

The aim of the work described in this report was to determine whether interspecies hydrogen exchange occurs between knallgas bacteria and cyanobacteria, and, if so, can knallgas bacteria be used as indicator organisms for the detection of cyanobacteria that overproduce hydrogen. Both culture-based and cultivation-independent approaches were used to investigate interactions between knallgas bacteria and cyanobacteria. For the culture-based approach, we tested for synergistic growth when cyanobacteria and knallgas bacteria were grown in co-culture or in vessels that allowed

gas exchange between axenic cultures. In addition, a reverse-transcriptase quantitative PCR method was developed to measure interspecies hydrogen exchange between the knallgas bacterium *Alcaligenes hydrogenophilus* and the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. For the cultivation-independent approach, stable isotope probing was used to detect knallgas bacteria in a variety of diverse ecosystems, particularly cyanobacteria dominated microbial mats.

INTERSPECIES HYDROGEN EXCHANGE BETWEEN A KNALLGAS BACTERIA AND CYANOBACTERIA

Background

Hydrogen exchange between cyanobacteria and knallgas bacteria was investigated using well-studied representatives from each group. The cyanobacteria used in this study were *Anabaena* sp. PCC7120 (PCC 7120) and *Synechocystis* PCC 6803. PCC 7120 is a filamentous, nitrogen-fixing cyanobacterium, and has been studied extensively for its ability to produce hydrogen (18, 19, 31, 45, 55, 63). PCC 7120 has an uptake hydrogenase that catalyses the consumption of most of the hydrogen produced under nitrogen fixing conditions (94). *Synechocystis* PCC 6803 is a well studied unicellular cyanobacterium that does not fix nitrogen (6, 7, 9, 26, 46, 47, 67, 87), but *Synechocystis* PCC 6803 has been shown to produce hydrogen transiently during the transition from a dark to light (26). Two uptake hydrogenase deficient mutants *Nostoc punctiforme* NHM5 (53) and *Anabaena* strain AMC 414 (20) were used as well.

The knallgas bacteria used in this study were *Cupriavidus necator* DSM 428 (Cn428) and *Alcaligenes hydrogenophilus* strain DSM 2625 (Ah2625). Cn428 is the most extensively studied representative of the knallgas bacteria, and its genome is sequenced (72). This organism is capable of utilizing both organic compounds and molecular H_2 as an energy source (72), either alternatively or concomitantly. Ah2625 was first isolated from soil (69), and is a hydrogen-oxidizing bacterium that possesses a membrane bound [NiFe] hydrogenase consisting of a small (*hupS*) and large (*hupL*) subunit (102). Unlike Cn428 (73), induction of the membrane bound hydrogenase in Ah2625 requires the presence of hydrogen (35, 102, 103) and the activity is repressed by organic substrates

(35, 101, 103). A low level of *hupS* expression has been observed when a poor carbon source is present (102, 103), suggesting that hydrogenase expression could occur under mixotrophic conditions. The above observations make Ah2625 an attractive candidate for evaluation of hydrogenase expression during autotrophic or mixotrophic growth conditions and as a potential biosensor for the presence of hydrogen.

Results

Synergistic growth experiments

In order to determine the minimum H_2 concentration that can support chemolithotrophic growth in Cn428, cultures were inoculated into minimal media under various concentrations of H_2. A measurable increase in protein (mg/mL) was observed when the H_2 concentration in the headspace was above 1,000 ppmV, which is equivalent to 0.78 μM in the liquid culture. This value is close to the K_m observed in unplanted non-sterile soil (>1300 ppmV H_2) (84).

In order to test whether gas exchange between cyanobacteria and knallgas bacteria could enhance the growth of both organisms, experiments were carried out with an apparatus designated the double bottle. Two serum bottles of volume between 14 – 30 mL were connected with a glass tube, physically separating the two cultures but allowing gas transfer between them. Different pairs of cyanobacteria and knallgas bacteria were tested with the following variables.

Different headspace conditions:

- Open to exchange with air
- Sealed with air in the headspace
- Sealed with hydrogen in the headspace
- Sealed with a gas mixture of H_2:O_2:CO_2 (70:20:10)
- Sealed with N_2:CO_2 (90:10)

Different parameters:

- Dark/light cycles: 1h/1h, 12h/6h, 6h/6h, 12h/12h
- Different concentrations of inocula and inocula from different growth stages

- Different pH (6-11)
- Different concentrations of $NaHCO_3$ or Na_2CO_3 (0-30mM)
- With or without nitrogen compounds in the media for the cyanobacteria
- Incubation at 25 or 30°C with or without stirring
- With or without addition of Ni^{2+}

Although a wide range of parameters were tested, we did not observe significant growth relative to control treatments when the knallgas bacteria were paired with cyanobacteria. There are several possible explanations for the negative results. In the cultures that were grown in double-bottles that were not sealed, H_2 did not accumulate above the threshold required for chemolithotrophic growth. In the cultures that were grown in sealed double-bottles, oxygen accumulated and inhibited H_2 production from the hydrogenase and nitrogenase. Because the growth of knallgas bacteria in the double-bottle experiments appeared to be an inadequate measurement for demonstrating hydrogen exchange, a reverse transcriptase-quantitative PCR (RT-qPCR) assay that measures *hupS* expression in Ah2625 was developed to detect hydrogen consumption by Ah2625 when grown with PCC 7120. The advantages the PCR-based method has over measuring growth are increased sensitivity and the applicability of the PCR assay to mixed cultures.

Validation of RT-qPCR method

Experiments were conducted to confirm that the RT-qPCR assay would be a sufficient and sensitive method for measuring gene expression as a proxy for hydrogen uptake. Expression of Ah2625 *hupS* was examined by RT-qPCR of the mRNA, and gene expression was calculated relative to expression of *rpoB* and the 16S rRNA genes. Similar results were obtained with both but *rpoB* gave greater uniformity of expression under various growth conditions than 16S rRNA, therefore we present expression relative to *rpoB*. Relative expression of *hupS* measured by RT-qPCR was also estimated based on the specific activity of membrane bound hydrogenase (MBH) measured using a spectrophotometric assay (76, 80). Both assays indicated that when Ah2625 cells were

grown on succinate and then transferred to knallgas conditions, hydrogenase activity was detectable after 6 h and peaked after 48 h incubation (Fig. 2.1 and 2.2). Although growth continued after 48 h, expression levels decreased, probably due to nutrient limitation.

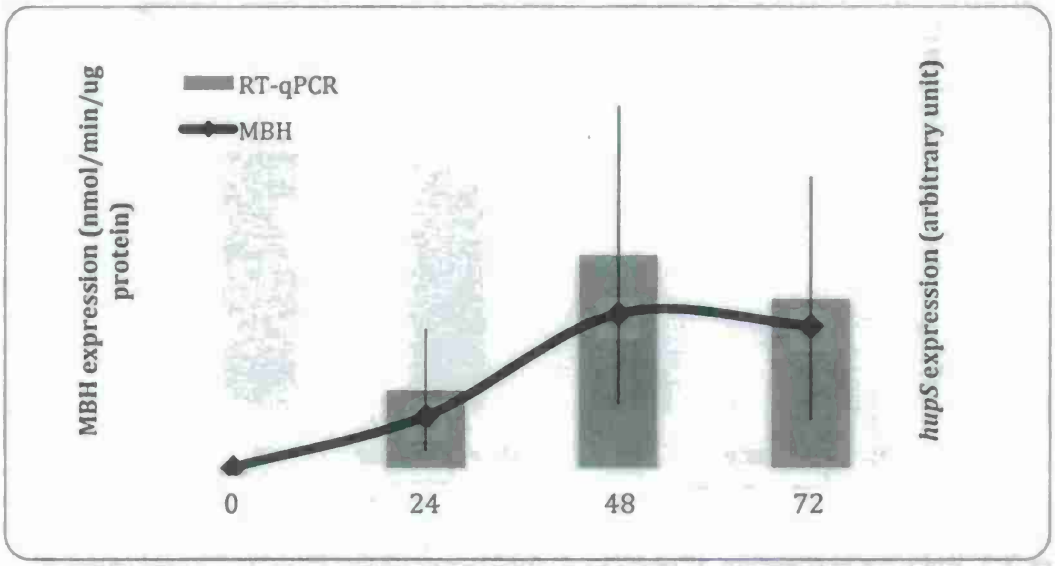

Figure 2.1. Relative expression of *hupS* hydrogen uptake gene of *A. hydrogenophilus* DSM 2625 grown under a gas mixture of approximately 60:10:10 H_2:O_2:CO_2 over a 96 h period, measured using RT-qPCR targeting the *hupS* hydrogenase and spectrophotometric assays of membrane bound hydrogenase (MBH). Error bars show standard deviation.

Specificity of *hupS* expression

To determine whether *hupS* expression was specific to chemolithotrophic growth, Ah2625 cells were incubated with either hydrogen as the sole energy source and carbon dioxide as the carbon source, or a combination of hydrogen with succinate as a readily available carbon source. RT-qPCR results confirmed that the *hupS* gene is only up-regulated when hydrogen is the sole energy source (35, 102, 103) (Fig. 2.2). *hupS* expression in cells grown solely under hydrogen was highly up-regulated compared to expression in heterotrophically grown cells. When cells were incubated with both hydrogen and succinate, there was no increase in expression of *hupS* while succinate was present (6 h). After the succinate was exhausted however, the cells switched to hydrogen

as the energy source as indicated by *hupS* expression. MBH assays confirmed the results (data not shown).

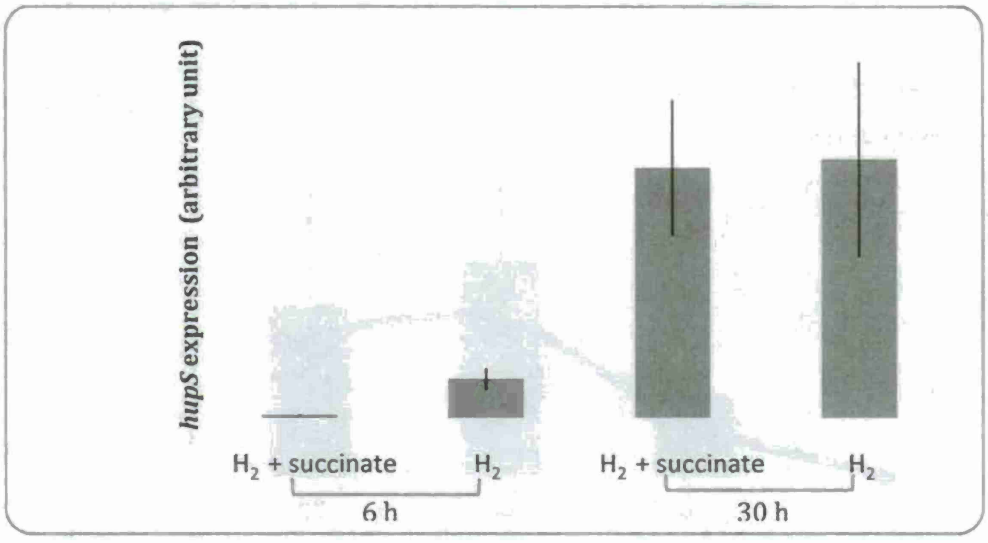

Figure 2.2. Relative expression of *hupS* in *A. hydrogenophilus* DSM2625 compared to *rpoB* expression, grown under a gas mixture of a gas mixture of approximately 60:10:10 H_2:O_2:CO_2, and with succinate added as a carbon source. Error bars shows standard deviation

Sensitivity of RT-qPCR assay

In order to determine the sensitivity of the RT-qPCR assay, *hupS* expression was measured when Ah2625 was grown under initial hydrogen headspace concentrations from 0.04 and 0.25 μM. The threshold hydrogen concentration that led to *hupS* up-regulation measurable by the RT-qPCR assay was between 0.04 and 0.12 μM (Fig. 2.3). The level of relative *hupS* expression did not appear to change substantially between an initial hydrogen concentration of 0.12 and 0.25 μM.

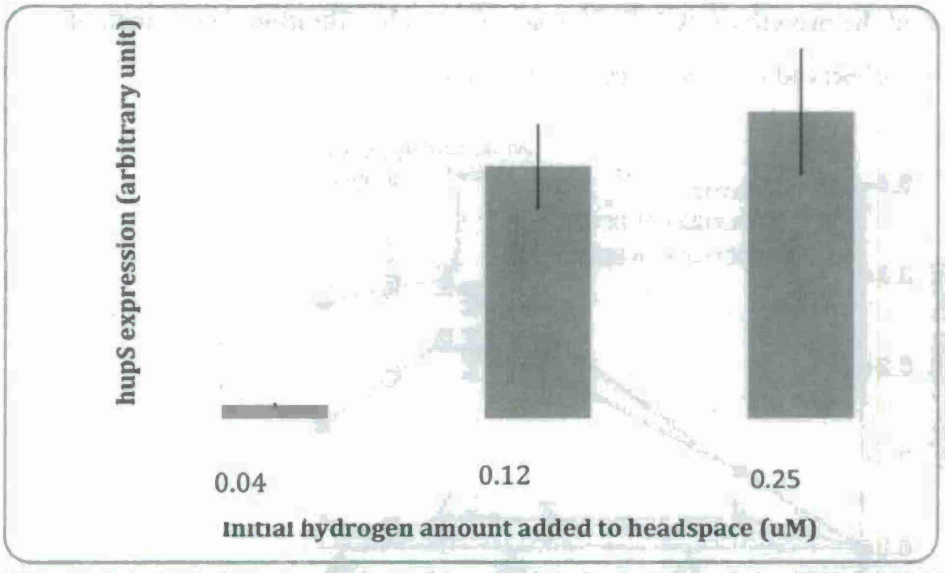

Figure 2.3. Relative expression of *hupS* of *A. hydrogenophilus* DSM 2625 compared to *rpoB* expression levels, grown under an air atmosphere with various concentrations of hydrogen added. Relative expression is compared to expression with no hydrogen added to cultures. Error bars represent standard deviation of at least three PCR reactions from duplicate bottles

Detection of hydrogen uptake in co-culture with hydrogen producing cyanobacteria

Once we confirmed that the RT-qPCR assay was effective and specific to chemolithotrophic growth, expression of the *hupS* gene in Ah2625 was tested in the presence of PCC 7120. PCC 7120 was incubated under an argon atmosphere which results in a higher rate of hydrogen production (55). The addition of Ah2625 substantially decreased hydrogen accumulation (Fig. 2.4). *hupS* expression was 398 (± 171) and 20 (± 13) times greater than at time zero, under an argon and air atmosphere respectively, confirming that Ah2625 was taking up the available hydrogen. Initial rates of hydrogen production were enhanced in the absence of O_2, but after 20 h sufficient oxygen had accumulated (data not shown) to inhibit hydrogen production and support the hydrogen oxidation by Ah2625. The results show that *hupS* up-regulation is a sensitive indicator of hydrogen exchange. No growth of Ah2625 was detected because of nitrogen limitation. Because we were concerned that Ah2625 was obtaining nutrients from organic exudates produced by the cyanobacterial cells, Ah2625 cells were incubated in a spent medium

that had supported the growth of PCC 7120 was removed by filtration. No growth of Ah2625 cells was observed after one week of incubation.

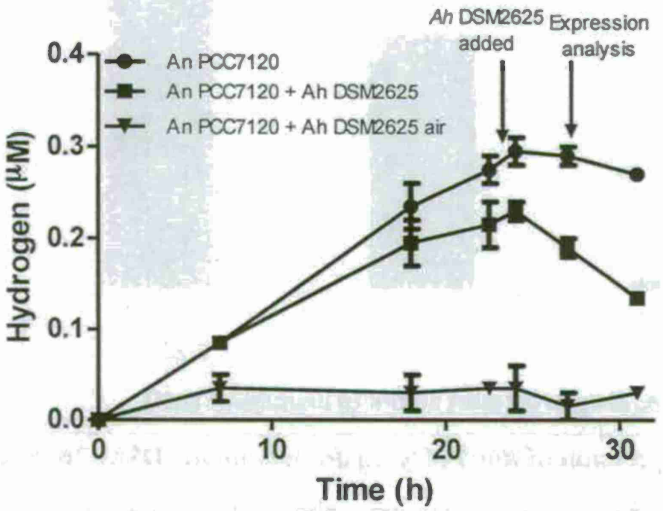

Figure 2.4. GC/TCD analyses of hydrogen concentrations in sealed serum bottles with an argon atmosphere containing either axenic cultures of *Anabaena* sp. PCC7120, *A. hydrogenophilus* DSM 2625, or a mixture of the two, where *A. hydrogenophilus* was added after 24 h of hydrogen production by *Anabaena* sp. PCC7120 and accumulation of hydrogen

To resolve concerns about differing nitrogen requirements and to determine whether the energy source for Ah2625 was a gas, cultures were physically separated using the double-bottles described above. The arrangement enabled supply of Ah2625 with ammonium but maintained nitrogen free conditions for PCC 7120, which can produce hydrogen under an argon atmosphere in the absence of nitrogen for up to 17 days (55). Ah2625 took up hydrogen when grown with PCC 7120 under an argon atmosphere (Fig. 2.5). Even though the initial atmosphere was argon photosynthesis produced oxygen during the incubation. Expression of *hupS* increased 24 (± 7.6) fold after 22 h when Ah2625 was present in the double bottle with PCC 7120. Expression of *hupS* under an air atmosphere was very low (2 ± 1.9 fold greater than at time zero). An increase in chlorophyll *a* (chl*a*) of PCC 7120 and oxygen accumulation indicated the culture was

active. These results clearly indicate that *hupS* up-regulation can be detected in real time, as hydrogen is being produced by the cyanobacterial cells.

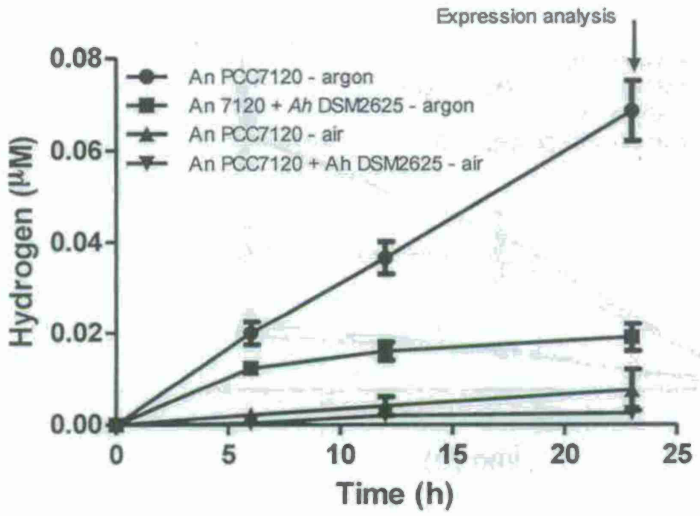

Figure 2.5. GC/TCD analyses of hydrogen concentrations in sealed double serum bottles with an argon atmosphere containing *Anabaena* sp. PCC7120 in one compartment, and either un-inoculated media or *A. hydrogenophilus* DSM 2625 in the other.

To avoid hydrogen consumption by the cyanobacteria in co-cultures, additional experiments were performed with AMC 414, a mutant *Anabaena* strain incapable of hydrogen uptake. AMC 414 produced hydrogen under both argon and air, and when Ah2625 was present in the second compartment of the double bottle, hydrogen accumulation was substantially lower (Fig. 2.6). Expression of the *hupS* gene was elevated 60 ± 2.4 fold after 12 h under an initial argon atmosphere. Under an air atmosphere *hupS* expression was also elevated (17 ± 4.7) although the hydrogen flux was not as dramatic, due to inhibition of hydrogen production in an air atmosphere (54). The results indicate that expression of *hupS* can be detected when hydrogen is produced by cyanobacteria over a range of concentrations. When AMC 414 and Ah2625 were both present at the beginning of the incubation period, *hupS* up-regulation was not detected, although GC/TCD analysis showed less hydrogen accumulation in the co-culture vessel. Oxygen accumulated in the headspace, and chl*a* levels of the cyanobacteria rose, indicating that the cells were active and likely producing hydrogen, which indicated that

hydrogen did not accumulate to levels sufficient to stimulate detectable expression of the enzyme.

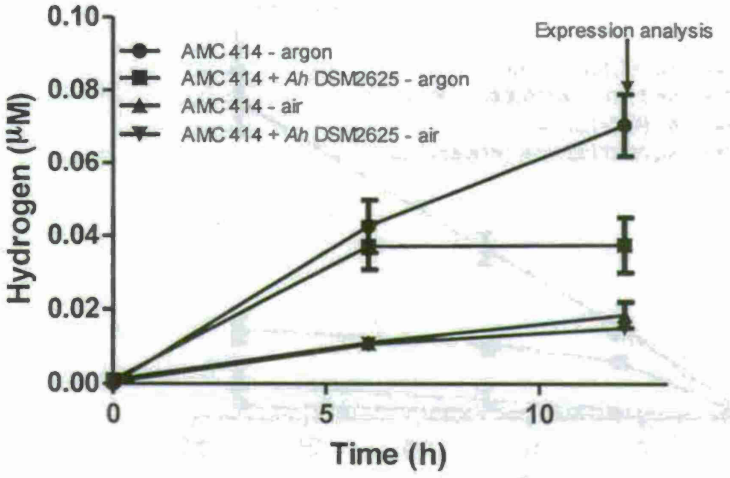

Figure 2.6. GC/TCD analyses of hydrogen concentrations in sealed double serum bottles with an argon atmosphere containing *Anabaena* strain AMC 414 in one compartment, and either un-inoculated media or *A. hydrogenophilus* DSM2625 in the other.

Conclusions

The purpose of this work was to detect hydrogen flux between cyanobacteria and knallgas bacteria by measuring expression of an uptake hydrogenase in knallgas bacteria. We used an RT-qPCR method for detecting *hupS* expression, which indicates hydrogen uptake in the hydrogen-oxidizing bacterium Ah2625, and confirmed previous studies (35, 102). The expression of *hupS*, which occurred only under conditions of autotrophic growth, is a sensitive indicator of hydrogen flux, and the assay can detect expression of *hupS* when hydrogen concentrations are similar to those found in hot springs (300 nM) and sewage sludge (203 nM) (89, 106). Expression of *hupS* was also up-regulated in the presence of hydrogen producing cyanobacteria, highlighting the potential for detecting hydrogen flux and interspecies hydrogen exchange in natural ecosystems where interactions would be expected to be highly evolved and specific (15, 39, 92). Synergistic relationships based on hydrogen exchange have been suggested to explain the presence of knallgas bacteria in environments with leguminous plant-associated N_2 fixers (51, 84)

and green algae. However to our knowledge an interaction based on hydrogen exchange between cyanobacteria and knallgas bacteria has not been demonstrated previously.

The fact that *hupS* expression was not detected when cells of Ah2625 were incubated from time zero with the hydrogenase mutant AMC 414 indicates that hydrogen did not accumulate to levels above the threshold for induction, possibly due to constitutive levels of hydrogenase activity. Although this suggests that the system would not be sufficiently sensitive for ecosystems where the atmosphere is the source of hydrogen, it should work well in ecosystems where hydrogen is already being produced. Even though hydrogen concentrations in the bulk medium of habitats might be minimal, there should be a substantial flux with higher concentrations near the producing organisms.

Additional refinements and development of the assay will allow investigation of the ecological roles and physiology of hydrogen-oxidizing bacteria in natural ecosystems including the rhizosphere and cyanobacterial mats. Future applications of the approach will include the use of knallgas bacteria to obtain a better understanding of hydrogen flux in natural environments, and as possible reporter strains to detect hydrogen producing organisms. The findings also suggest that transcriptomic profiles of microbial communities could be used to detect up-regulation of *hupS* homologs and indicate hydrogen exchange among uncultured members of the community (62).

CULTIVATION-INDEPENDENT DETECTION OF HYDROGEN-OXIDIZING BACTERIA

Background

Because knallgas bacteria are a physiologically defined group, their identification has primarily relied on cultivation. Given that current cultivation techniques fail to grow most bacteria, reliance on cultivation certainly limits our understanding of knallgas bacteria in the natural world. Cultivation-independent techniques that selectively detect and identify knallgas bacteria will improve our ability to study their diversity, distribution, and ecology. Stable isotope probing (SIP) is a cultivation independent technique that links function and identity (68), and it has already been used to identify

chemolithoautotrophs including ammonia-oxidizing bacteria, iron-oxidizing bacteria, and sulfur-oxidizing bacteria and archaea (22, 37, 38, 49, 99, 105). Here SIP was used to identify knallgas bacteria. First, to demonstrate the technique, knallgas bacteria were identified from the rhizosphere of legumes. Hydrogen is a by-product of nitrogen fixation in root nodules (83), and if the N_2-fixing symbionts are deficient in uptake hydrogenase the H_2 is released to the surrounding soil. Dong and Layzell calculate that soil 1-4 cm from N_2-fixing root nodules is exposed to H_2 at a rate of 30-254 nmol cm^{-3} hr^{-1} (30), and both hydrogen consumption and the MPN of knallgas bacteria decreased exponentially with distance from root nodules (51). After demonstrating the feasibility of identifying knallgas bacteria with SIP, the technique was applied to cyanobacteria-dominated microbial mats to investigate the potential association between knallgas bacteria and cyanobacteria in natural habitats.

Results

SIP of knallgas bacteria in rhizosphere soil

Initial experiments were conducted with soil incubated under 800 ppm hydrogen, which is near the reported K_m for hydrogen uptake by many knallgas bacteria and soil (84). The headspace hydrogen concentration was monitored to determine whether H_2 was consumed during SIP incubations with soil from the rhizosphere of white clover and vetch plants. The two soils displayed different H_2 uptake activity (Fig. 3.1), with the vetch soil taking nearly twice as long to consume the same amount of H_2 as the white clover soil. The maximum H_2 uptake rates observed for the white clover soil under $^{12}CO_2$ + H_2 or $^{13}CO_2$ + H_2 were 0.50 μmoles H_2 hr^{-1} g $soil^{-1}$ and 0.46 μmoles H_2 hr^{-1} g $soil^{-1}$, respectively. The corresponding rates for the vetch soil were 0.21 μmoles H_2 hr^{-1} g $soil^{-1}$ and 0.17 μmoles H_2 hr^{-1} g $soil^{-1}$, respectively. The H_2 uptake indicated that H_2-oxidizing bacteria were active in both rhizosphere soils, and the increasing rates suggest growth of the populations.

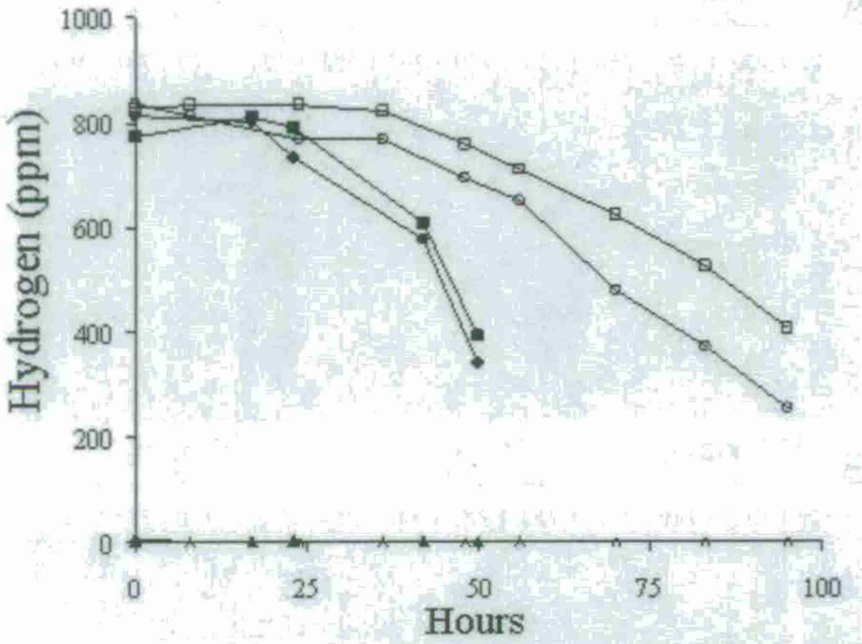

Figure 3.1. Analysis of the hydrogen concentration during incubations of white clover rhizosphere soil (filled symbols), vetch rhizosphere soil (open symbols). The data points represent the mean hydrogen concentration for duplicate measurements of $^{13}CO_2$ without H_2 (triangles), $^{12}CO_2$ with H_2 (circles), and $^{13}CO_2$ with H_2 (squares) treatments.

To determine whether incorporation of the ^{13}C-label accompanied the H_2 uptake observed with rhizosphere soil, 16S rRNA gene profiles from CsCl gradient fractions were compared by DGGE (Fig. 3.2). In all samples and treatments, a complex 16S rRNA gene profile was present in the less dense fractions (1.718-1.732 g ml^{-1}), which contain the unlabeled DNA. In contrast, in the fractions with a density of 1.736-1.754 g ml^{-1}, no bands or only faint bands were detected when samples were incubated without H_2 or with $^{12}CO_2$ or ^{12}C-bicabonate. Unique 16S rRNA gene profiles were only detected in the dense fractions when samples were given both $^{13}CO_2$ and H_2. The bands that are present in the dense fractions of the $^{13}C + H_2$ treatment, but are absent from comparable fractions in the control treatments, represent knallgas bacteria.

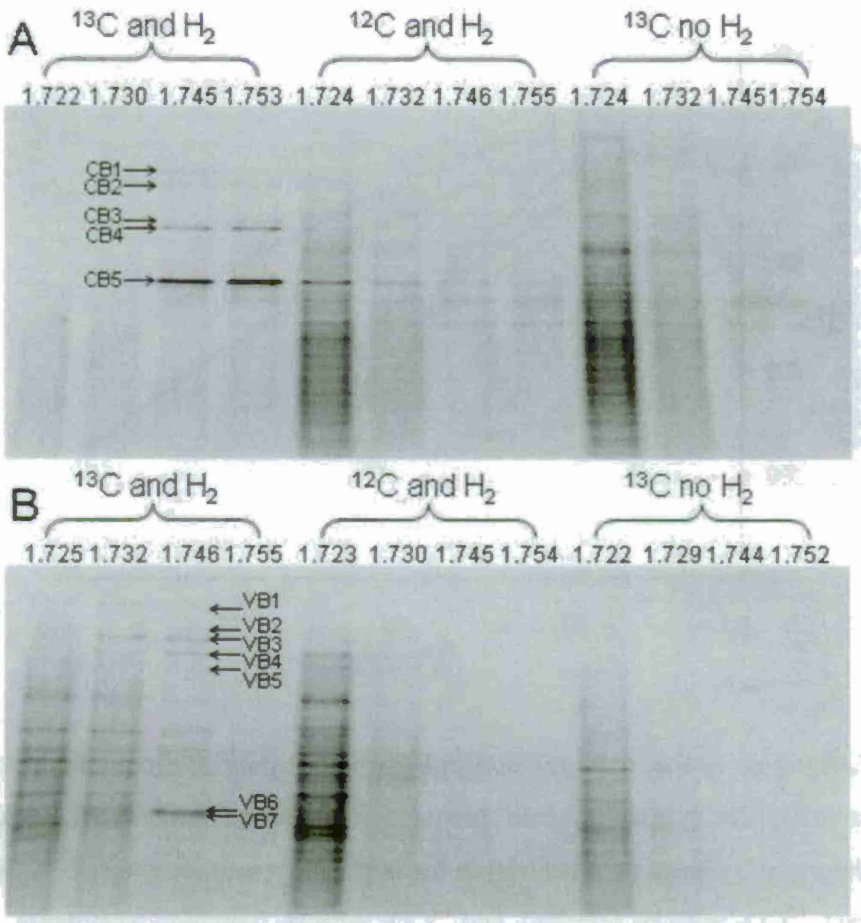

Figure 3.2. DGGE profiles of 16S rRNA genes amplified from CsCl gradient fractions following isopycnic centrifugation of DNA extracted from (A) white clover rhizosphere soil, (B) vetch rhizosphere soil. The value above each lane indicates the density (g ml^{-1}) of that fraction.

SIP of knallgas bacteria in microbial mats

After determining that the SIP incubations with soil were successful and ^{13}C-labeled DNA could be detected, SIP incubations were conducted with samples from cyanobacteria-dominated microbial mats. As with the soil, the headspace hydrogen concentration was monitored to determine whether H_2 was consumed during SIP incubations with the microbial mats. Hydrogen uptake was observed for all samples tested (example Figure. 3.3; see appendix for additional figures of H_2 uptake by microbial mats), suggesting that H_2-oxidizing bacteria were active. The majority of the mats tested

consumed most of the H_2 in 2 to 3 days, but the mat grown in the laboratory aquarium only consumed 30% of the hydrogen in 7 days. When incubated under 1000 ppm H_2, uptake rates ranged from 0.11 to 0.25 μmoles H_2 hr^{-1} g $sample^{-1}$, which is comparable to the rates observed in the soil samples.

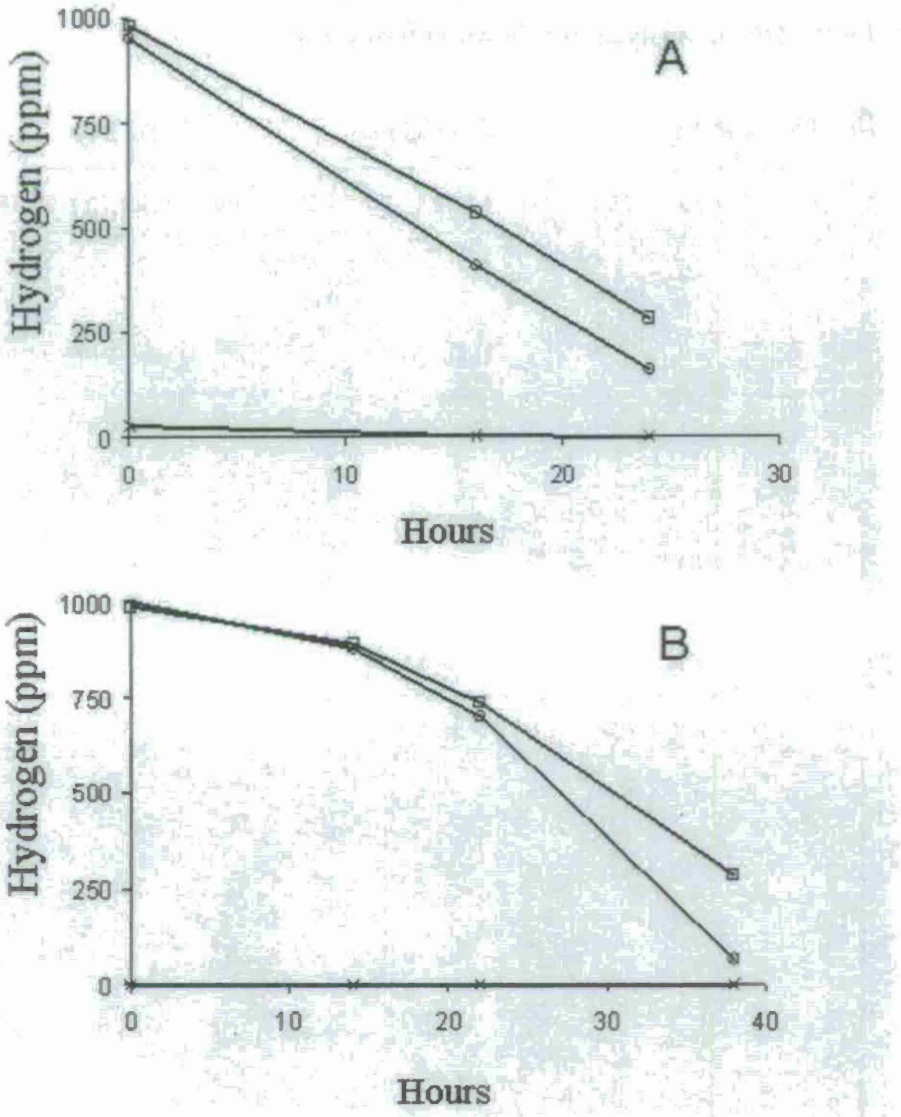

Figure 3.3. Analysis of the hydrogen concentration during incubations with the top green layer from microbial mats Elkhorn Slough 1 (A) and Elkhorn Slough 2 (B). The data points represent the mean hydrogen concentration for duplicate measurements of $^{13}CO_2$ without H_2 (×), $^{12}CO_2$ with H_2 (○), and $^{13}CO_2$ with H_2 (□) treatments.

Although the microbial mats consumed H_2, DGGE analysis of density resolved DNA revealed that ^{13}C-labeled DNA was only detected in microbial mats collected from Obsidian Pool (Fig 3.4). The absence of ^{13}C-labeled DNA in the other microbial mat samples suggests that heterotrophic hydrogen-oxidizing bacteria or cyanobacteria were responsible for the observed hydrogen uptake. Representative examples of negative results for the DGGE analysis are shown in figure 3.5.

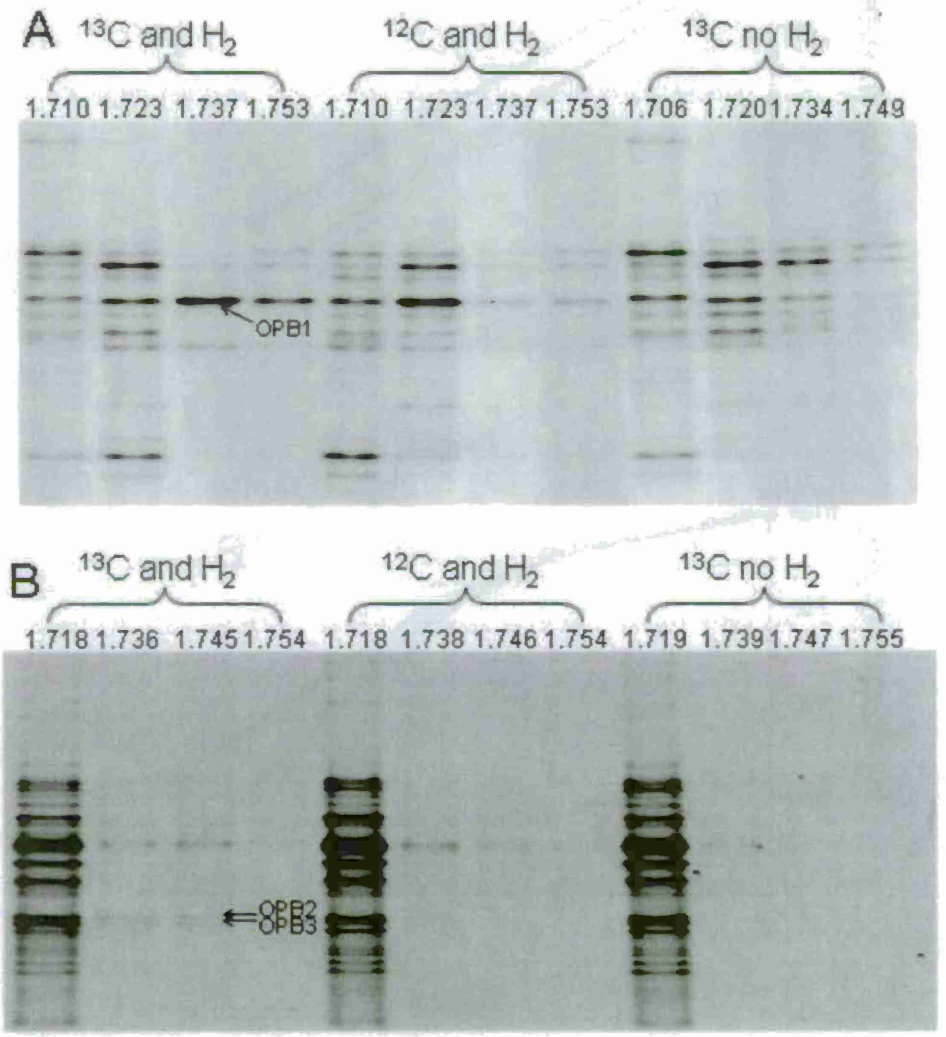

Figure 3.4. DGGE profiles of 16S rRNA genes amplified from CsCl gradient fractions following isopycnic centrifugation of DNA extracted from (A) Obsidian Pool 1 and (B) Obsidian Pool 2. The value above each lane indicates the density (g ml^{-1}) of that fraction. Arrows indicate bands selected for sequence analysis.

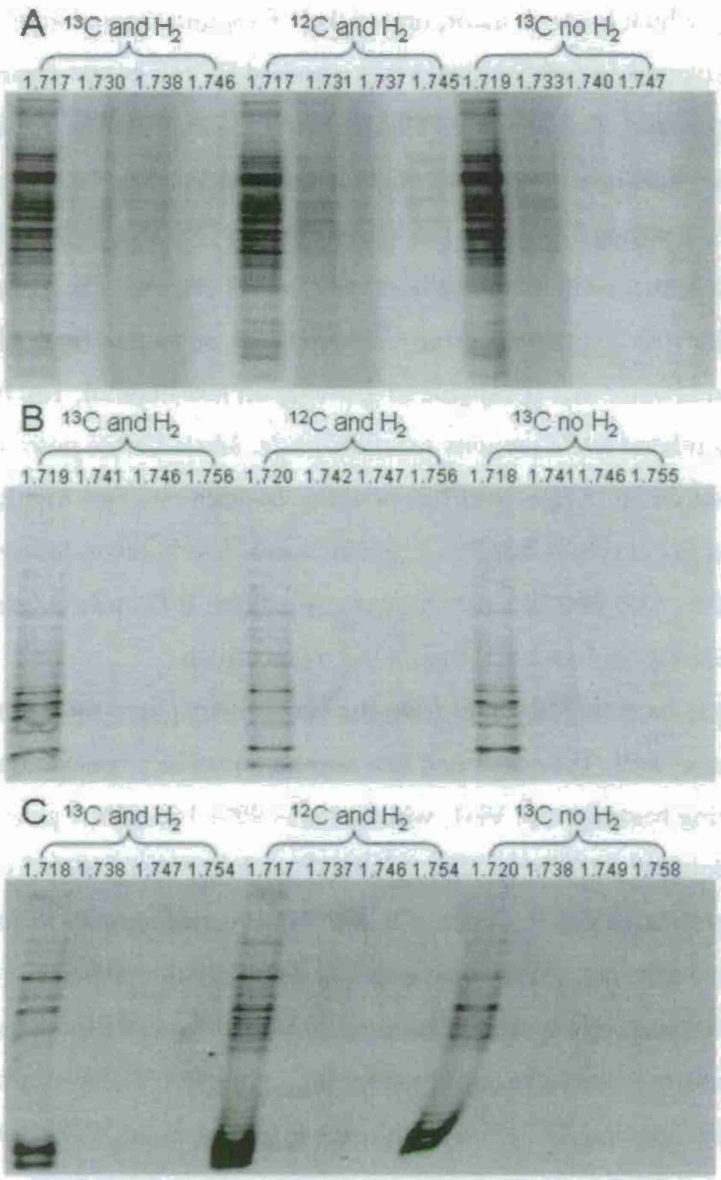

Figure 3.5. Representative negative results for DGGE analysis of 16S rRNA genes amplified from CsCl gradient fractions following isopycnic centrifugation of DNA extracted from (A) Everglades EC2T, (B) Elkhorn Slough 2, and (C) Sperm Pool 1. The value above each lane indicates the density (g ml^{-1}) of that fraction..

Identification of knallgas bacteria

To identify which bacteria incorporated the $^{13}CO_2$ into their biomass, selected DGGE bands and cloned 16S rRNA genes that matched DGGE bands present in "heavy" fractions were sequenced. The identified knallgas bacteria are listed in Table 3.3.

Five betaproteobacteria were identified as knallgas bacteria from the white clover rhizosphere soil. The strongest band in the heavy fraction DGGE profile, CB5, was also the most abundant sequence in the clone library (25 of 48 clones). The gene sequence shares 97% identity with *Aquincola tertiaricarbonis* L10, an isolate from MTBE-contaminated groundwater that is capable of growing on *tert*-butanol. The other strains were most closely related to *Pelomonas saccharophila*, *Methylibium petroleiphilum*, *Ideonella dechloratans*, and *Aquaspirillum articum*. In addition, Two knallgas bacteria were isolated from the clover rhizosphere, and both are closely related to knallgas bacteria identified by SIP. The 16S rRNA genes of isolates C92 and C93 share 99% and 99.7% sequence identity to bands CB4 and CB5, respectively.

The knallgas bacteria identified from the vetch rhizosphere soil were primarily betaproteobacteria as well. The only band that corresponded to a species known to contain H_2-oxidizing bacteria was VB1, which shares 99% 16S rRNA gene sequence identity with *Variovorax paradoxus*. The sole knallgas bacterium isolated from the vetch rhizosphere possesses a 16S rRNA gene with 99.9% sequence identity to band VB1. The predominant cloned sequences from the vetch soil corresponded to bands VB3 (13 of 48) and VB4 (12 of 48), and were closely related to *Polaromonas rhizosphaerae* and *Methylibium fulvum*, respectively. The closest relative of band VB5 was also a *Methylibium fulvum* species. The cloned sequence matching band VB7 shares 97% identity with *Aeromicrobium ginsengisoli*, an actinomycete isolated from a ginseng field (48). No cloned sequence matched band VB6, but the sequence of the extracted DGGE band shares 98% identity with *Aeromicrobium ginsengisoli*.

There were three bacteria identified from the two Obsidian Pool samples. The sequence matching the single band from Obsidian Pool 1, OPB1, shares 96% identity with *Schegelella thermodepolymerans* strains KA1 and SA1, isolates from hot compost. There were two bands enriched in ^{13}C from the Obsidian Pool 2 microbial mat. Based on 16S rRNA gene similarity, the organism represented by band OPB3 is a member of the

alphaproteobacteria and is closely related to *Magnetospirillum bellicus* strain VDY, a strain isolated for its ability to reduce perchlorate. The sequence of the band OPB2 shares 99% sequence identity with *Spirochaeta caldaria* strain DSMZ7334. The organisms represented by bands OPB2 and OPB3 are closely related to bacteria known to oxidize H_2 under anaerobic conditions (64, 93), which suggests that anaerobic conditions developed in the mat during the SIP incubation even though the O_2 concentration in the headspace was never below 11%.

Table 1

Phylogenetic affiliation of partial bacterial 16S rRNA gene sequences corresponding to prominent bands identified by DGGE and isolated bacteria.

Sample Source	DGGE Band	Isolate	Closest Relative	Percent Identity	
				Band	Isolate
White Clover					
	CB1		*Aquaspirillum articum* (AB074523)	96	
	CB2		*Ideonella dechloratans* (GU168990)	97	
	CB3		*Methylibium petroleiphilum* (AF176594)	98	
	CB4	C92	*Pelomonas saccharophila* (AM501428)	99	98
	CB5	C93	*Aquincola tertiaricarbonis* (DQ656489)	97	97
Hairy Vetch					
	VB1	VB55	*Variovorax paradoxus* (AB552859)	99	99
	VB3		*Polaromonas rhizosphaerae* (EF127651)	99	
	VB4		*Methylibium fulvum* (AB245357)	97	
	VB5		*Methylibium fulvum* (AB245357)	98	
	VB6		*Aeromicrobium ginsengisoli* (FR682667)	98	
	VB7		*Aeromicrobium ginsengisoli* (AB245395)	97	
Obsidian Pool 1					
	OPB1		*Schegelella thermodepolymerans* (AY538709)	96	
Obsidian Pool 2					
	OPB2		*Spirochaeta caldaria* (EU580141)	99	
	OPB3		*Magnetospirillum bellicus* (EF405824)	98	

Hydrogen concentration threshold for detection by SIP

To test whether SIP could be used to discriminate between knallgas bacteria with different H_2 uptake thresholds, SIP incubations with the white clover and vetch soils were conducted under H_2 concentrations of 500, 250, and 100 ppm. Although H_2 uptake was observed at all concentrations, H_2 uptake kinetics differed between the two soils (Fig. 3.6). The patterns of H_2 uptake at 500 ppm and 800 ppm were similar and the clover soil consumed H_2 more quickly than the vetch soil. At 100 ppm H_2 the pattern was reversed and the vetch soil consumed H_2 more quickly, suggesting that knallgas bacteria from the vetch soil are better adapted for H_2 uptake at lower concentrations.

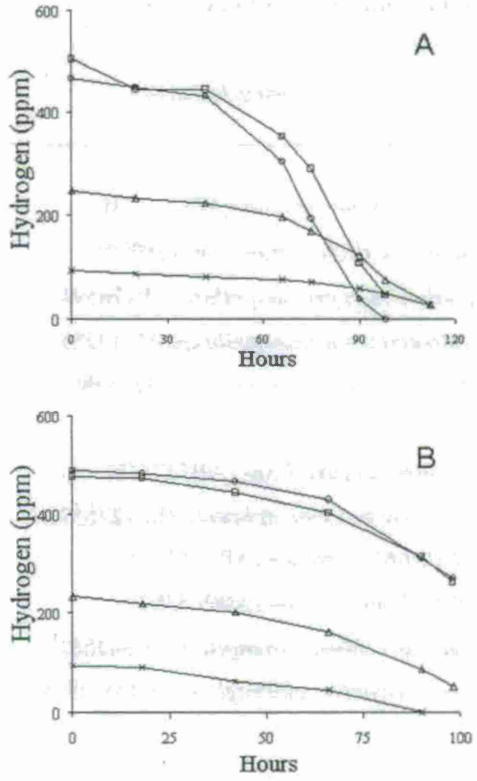

Figure 3.6. Analysis of the hydrogen concentration during incubations of (A) white clover rhizosphere soil and (B) vetch rhizosphere soil. The data points represent the mean hydrogen concentration for duplicate measurements of $^{12}CO_2$ with 500 ppm H_2 (○), $^{13}CO_2$ with 500 ppm H_2 (□), $^{13}CO_2$ with 250 ppm H_2 (Δ), and $^{13}CO_2$ with 100 ppm H_2 (×) treatments.

DGGE analysis of CsCl gradient fractions revealed that knallgas bacteria could be detected by SIP when the soils were incubated under 250-800 ppm H_2 (Fig. 3.7). When the soils were incubated under 100 ppm H_2, only faint ^{13}C-DNA bands were detected from the white clover soil and only bands VB6 and VB7 were detected from the vetch soil. The detection of the *Aeromicrobium* species and the absence of the betaproteobacteria at 100 ppm H_2 suggest that the actinomycetes are better adapted for growth at low H_2 concentrations.

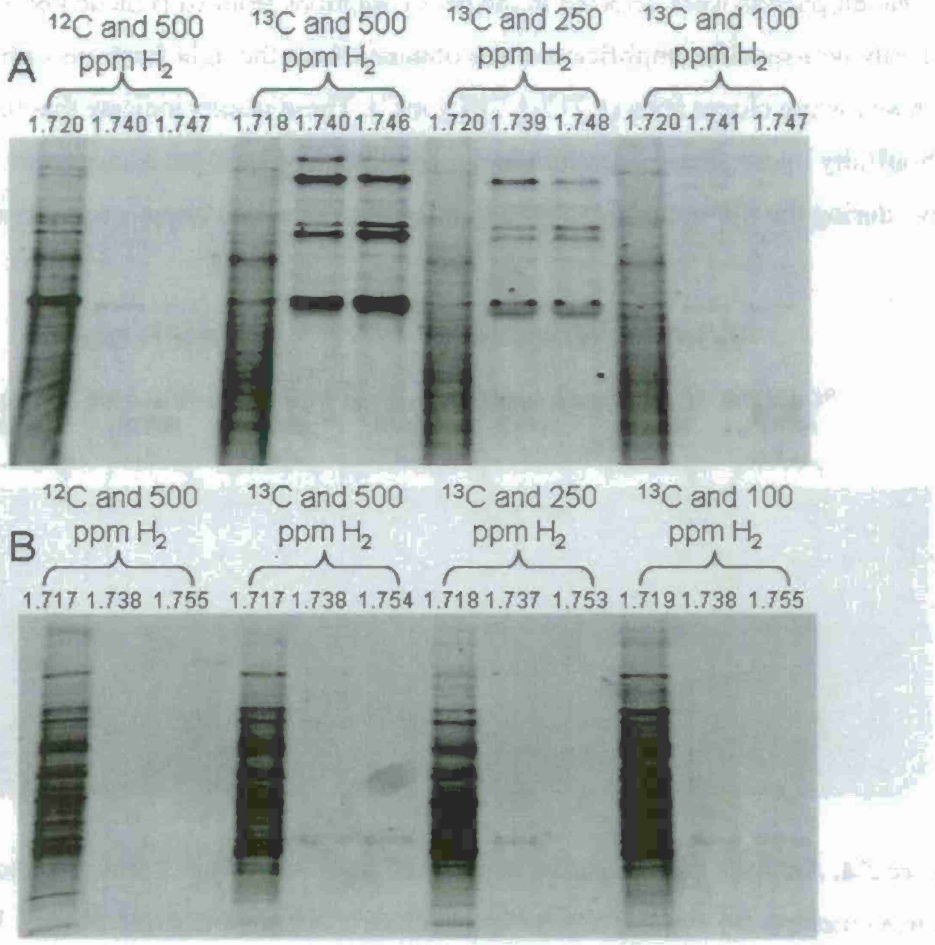

Figure 3.7. DGGE profiles of 16S rRNA genes amplified from CsCl gradient fractions following isopycnic centrifugation of DNA extracted from (A) white clover rhizosphere soil and (B) vetch rhizosphere soil incubated under 500, 250 or 100 ppm H_2. The value above each lane indicates the density (g ml^{-1}) of that fraction.

Amplification of *hydB* hydrogenase genes from ^{13}C-labeled DNA

Because the actinomycetes detected in the vetch soil appear to grow well even at the lowest H_2 concentration tested, primers designed to amplify the *hydB*-like gene were used to test for the presence of the putative high-affinity hydrogenase that has been found in species of *Streptomyces*. Even though the actinomycetes were only detected in the vetch soil, a ~1.4 kb *hydB*-like amplicon was detected in the heavy fractions from the treatments that received H_2 and $^{13}CO_2$ from both the vetch and white clover soils (Fig. 3.8). No amplicons were detected in the heavy fractions from soils incubated with $^{12}CO_2$. Primarily non-specific amplification was obtained from the light fractions of both the vetch and white clover soils (1.717-1.719 g ml^{-1}). These results indicate that the putative high-affinity hydrogenase is present in the population of knallgas bacteria that were active during the SIP incubations though it is not clear which organisms harbor the gene.

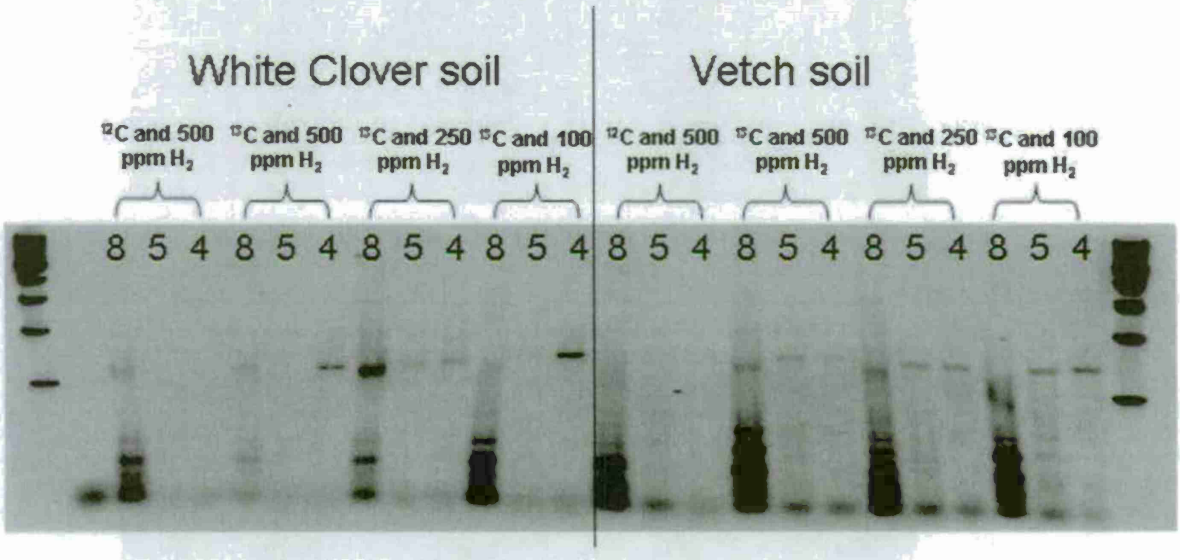

Figure 3.8. Analysis of PCR products obtained from white clover and vetch soil with primers targeting the *hydB*-like gene by gel electrophoresis (negative image). The numbers above each lane indicate which gradient fraction the template was from. 8 = buoyant density of 1.717-1.720 g ml^{-1}, 5 = buoyant density of 1.737-1.741 g ml^{-1}, and 4 = 1.745-1.748 g ml^{-1}. A 1 kb DNA step ladder was used as the marker.

Phylogenetic analysis of the amino sequences of *hydB*-like genes that were cloned from the two soils revealed that the hydrogenases cluster according to the soil from which

they were amplified (Fig 3.9). Hydrogenases from the white clover soil cluster closely to the NiFe-hydrogenases found in the *Streptomyces* species that can oxidize tropospheric H_2. The *hydB*-like hydrogenases from the vetch soil cluster with hydrogenases that are primarily associated with uncultured bacteria.

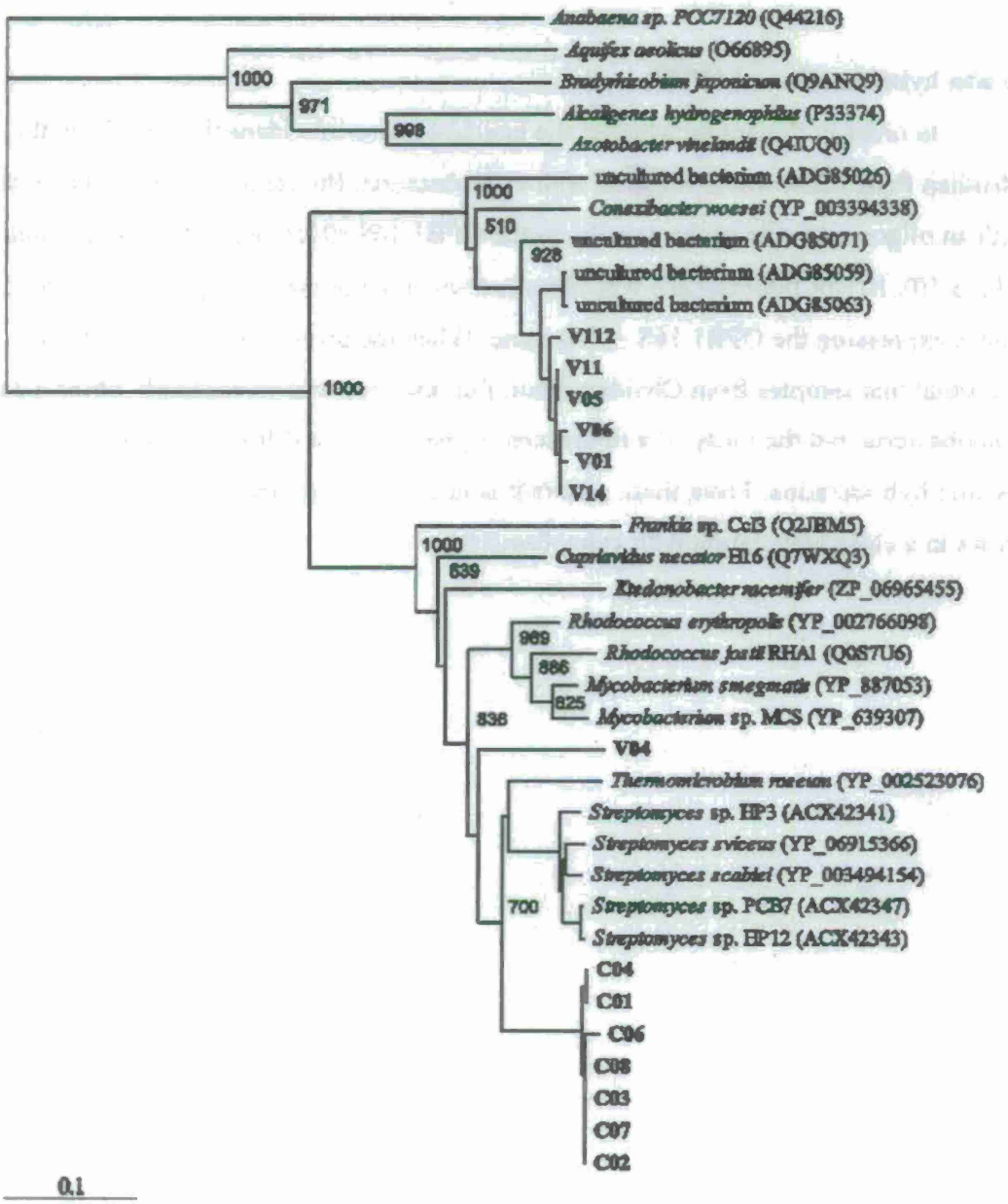

Figure 3.9. Phylogenetic tree of partial amino acid sequences translated from hydB-like gene sequences that were cloned from the "heavy" SIP fractions from vetch and clover soil (bold), and gene sequences for large subunits of NiFe-hydrogenase available in public databases. Cloned sequences with names beginning with C were amplified from the clover soil, and those beginning with V were amplified from the vetch soil. The scale bar represents 10% sequence divergence.

In situ hybridization of OPB1

In order to determine whether the knallgas bacterium identified by SIP in the Obsidian Pool 1 mat was associated with cyanobacteria, fluorescent in situ hybridization with an oligonucleotide probe specific to the OPB1 16S rRNA sequence was carried out (Fig. 3.10). Bright fluorescence was observed when the probe was hybridized with *E. coli* clones expressing the OPB1 16S rRNA gene. When the probe was applied to fixed microbial mat samples from Obsidian Pool, fluorescence was occasionally observed near cyanobacteria, but the rarity of a fluorescent signal makes it difficult to rule out non-specific hybridization. From these results it is not possible to determine whether OPB1 grows in a close association with cyanobacteria.

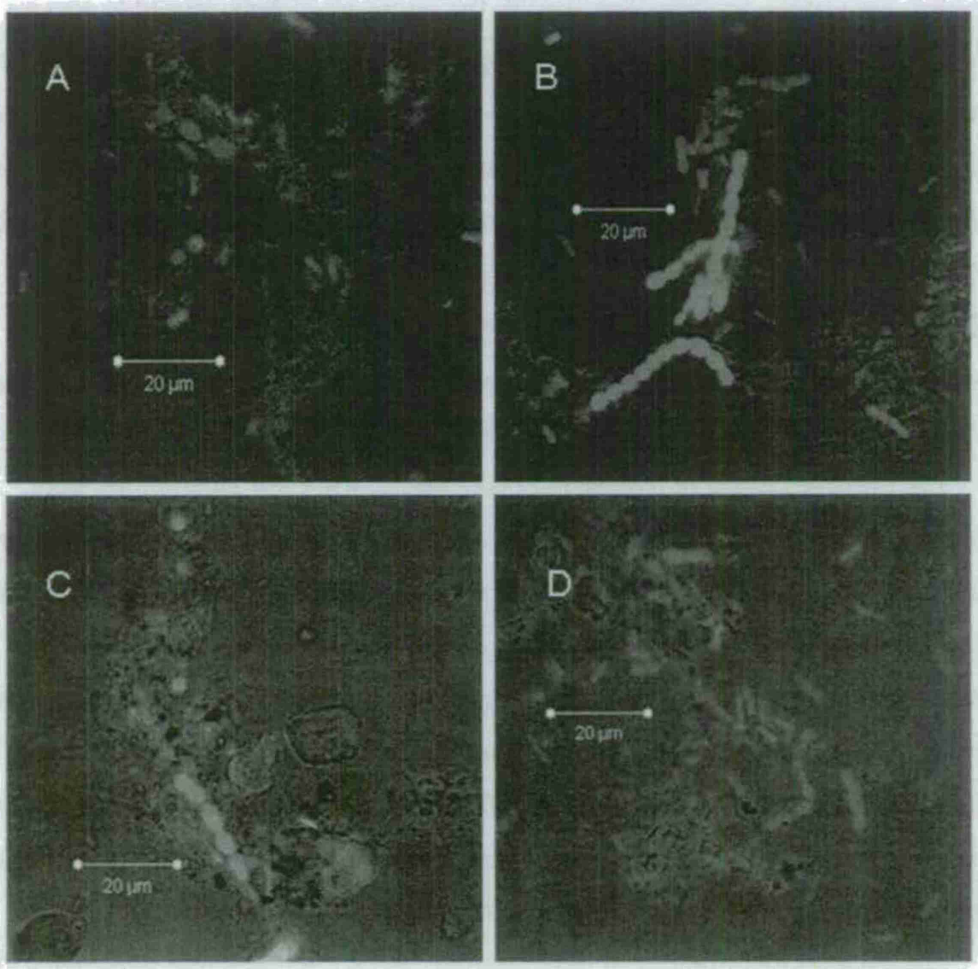

Figure 3.10. Visualization of the Obsidian Pool 1 microbial mat by laser confocal microscopy. The mat samples were hybridized with a positive control EUB probe (A), a negative control nonsense probe (B), and a probe targeting the OPB1 16S gene sequence (C and D).

Conclusions

The results presented here indicate that knallgas bacteria can be identified by SIP. Analysis of 16S rRNA gene profiles from CsCl gradient fractions revealed that incorporation of $^{13}CO_2$ occurred when soils were provided with H_2, but in the absence of H_2, endogenous electron donors did not support autotrophic growth sufficient to label DNA with ^{13}C. All but two of the knallgas bacteria identified in the rhizosphere soil were betaproteobacteria, with the actinomycetes represented by bands VB6 and VB7 being the

exceptions. Two of the bacteria identified in this study are closely related to species known contain knallgas bacteria *P. saccharophila, V. paradoxus* (11), which supports the conclusion that the other bacteria detected by SIP were knallgas bacteria. Furthermore, although chemolithotrophic growth on H_2 has not been documented in *P. rhizosphaerae*, both *P. hydrogenivorans* and *P. naphthalenivorans* CJ2 can grow chemolithotrophically with H_2 (86). The detection of *hydB* genes shows that functional genes involved in hydrogen metabolism can be detected by SIP as well, and suggests that cultivation-independent detection of O_2-tolerant hydrogenases could be accomplished by designing SIP experiments that select for O_2-tolerant knallgas bacteria.

The observed range of hydrogen uptake rates during SIP incubations were comparable to previously published V_{max} values, 0.01-0.4 μmoles H_2 hr^{-1} g $soil^{-1}$, for the oxidation of H_2 by soil (24). However, there were significant differences in H_2 uptake kinetics between the two soils. When the H_2 concentration was above 100 ppm, the difference in H_2 uptake by the microbes in the clover and vetch rhizosphere soil was likely due to faster growth of the knallgas bacteria in the clover soil. The difference in H_2 uptake by the microbes in the two soils at 100 ppm H_2 can be attributed, in part, to the presence of the actinomycetes in the vetch soil, because DGGE analysis revealed that incorporation of $^{13}CO_2$ at 100 ppm H_2 was limited to bands VB6 and VB7.

The bacteria identified from the Obsidian Pool 2 microbial mat sample are closely related to organisms known for anaerobic growth. To minimize disturbance of the microbial mat the samples were not shaken, thus anaerobic microenvironments could have developed. Band OPB2 is likely from a spirochete, and acetogenic growth on H_2 and CO_2 has been reported for spirochetes (64), while aerobic H_2-oxidation has not. Although preliminary, this may be the first example of a hydrogenotrophic acetogen detected by SIP. Monosaccharide-consuming and propionate-oxidizing acetogens have been detected previously (60, 78), but further work is needed to determine whether SIP could be a useful tool for the study of hydrogenotrophic acetogens. The organism corresponding to band OPB3 is closely related to *M. bellicus* strain VDY, which can grow chemolithotrophically with H_2 as an electron donor using perchlorate as an electron acceptor (93), and although strain VDY can grow aerobically, aerobic growth on H_2 has not been reported. Therefore our results cannot conclusively determine whether the

growth of OPB3 was aerobic or anaerobic. The results support the conclusion that SIP enabled the detection of bacteria that incorporated the ^{13}C-labeled substrate, and the incubation conditions determine whether aerobic or anaerobic H_2-oxidizing chemolithotrophs are detected.

Overall, SIP of knallgas bacteria does not appear to be suited to microbial mat samples, because ^{13}C-labeled DNA was rarely detected even though the cyanobacteria-dominated microbial mats consumed H_2 rapidly during SIP incubations. One possible reason for these observations is that H_2 oxidation is not limited to knallgas bacteria and uptake hydrogenase activity in heterotrophic N_2-fixing bacteria or cyanobacteria can compete with knallgas bacteria for H_2 during SIP incubations. Heterotrophic N_2-fixing bacteria are commonly found in marine microbial mats and may be responsible for the H_2 uptake observed in the mats we tested (70, 104). Similarly, almost all N_2-fixing cyanobacteria possess an uptake hydrogenase (17) and couple H_2 uptake with N_2-fixation (100). Under low oxygen and low light conditions, some cyanobacteria are able to use H_2 as an electron donor to fix CO_2 in a process called photoreduction (32, 34). However, CO_2 fixation and growth with H_2 as an energy source is not expected to be significant for cyanobacteria incubated the dark (32).

If knallgas bacteria were present in the microbial mats in which no ^{13}C-DNA was detected, then they were not growing autotrophically during the SIP incubations. Because many knallgas bacteria are facultative autotrophs or can grow mixotrophically on hydrogen, SIP may not be appropriate for detecting knallgas bacteria in samples with high levels of organic carbon. Transfer of carbon from cyanobacteria to heterotrophic bacteria has been demonstrated by nanoSIMS (14), and organic carbon provided by cyanobacteria could repress autotrophic growth by knallgas bacteria. If the knallgas bacteria consume endogenous organic carbon in addition to the labeled CO_2, the amount of ^{13}C-label in the DNA would be insufficient to distinguish labeled and unlabeled DNA by isopycnic centrifugation. Therefore, in addition to measuring H_2 uptake, measurements of $^{13}CO_2$ in the headspace should be considered to provide evidence of autotrophic growth.

However, the methods described here should be applicable to many other ecosystems, such as soil, marine, and geothermal systems. For example, SIP could

complement studies of the interaction between plants and knallgas bacteria populations and reveal whether certain plants increase carbon fixation in soils by promoting the growth of knallgas bacteria. Further work is needed to determine whether knallgas bacteria that oxidize H_2 at tropospheric levels can be detected by SIP. It is likely that longer incubations will be necessary, because ^{13}C-DNA was not detected in the controls incubated under ambient air without additional H_2.

OVERALL CONCLUSIONS AND RECOMMENDATIONS

The aim of the work described in this report was to determine whether interspecies hydrogen exchange occurs between knallgas bacteria and cyanobacteria, and whether knallgas bacteria would be suitable indicator organisms for the detection of cyanobacteria that overproduce hydrogen. The results suggest that although hydrogen exchange between cyanobacteria and knallgas bacteria is possible the effectiveness of knallgas bacteria as indicators of hydrogen producing cyanobacteria may be limited because our understanding of H_2 flux in microbial communities is incomplete. H_2 uptake was observed for almost every sample tested, but knallgas bacteria were rarely identified as the H_2 oxidizers, indicating that other members in microbial community were consuming H_2. In addition, measuring the expression of *hupS* in Ah2625 was an effective indicator of hydrogen exchange in vitro, but the lack of detectable *hupS* expression when Ah2625 and AMC 414 were grown together indicates more research is needed to determine if this approach can be adapted to natural systems. The lack of *hupS* expression may have been due to constitutive hydrogenase activity, or it is possible that the presence of Ah2625 inhibited H_2 production by AMC 414.

The potential for other bacteria and other hydrogenases to consume H_2 suggests a wider net be cast to better understand H_2 flux in microbial systems. One possible approach would be to design a microarray chip that contains probes for genes involved in hydrogen metabolism, such as hydrogenase and nitrogenase genes, to provide a transcriptomic profile of H_2 producers and consumers in a microbial community. A similar approach has been used to investigate interspecies H_2 exchange during reductive dehalogenation (62), and this could facilitate discovery of bacteria that contribute to hydrogen flux in cyanobacteria-dominated ecosystems. Furthermore, using an isotope

array approach (2), it may be possible to detect cyanobacteria that express nitrogenase or hydrogenase when growing under light.

EXPERIMENTAL PROCEDURES

Culture conditions

Anabaena sp. PCC 7120 was obtained from the Pasteur Culture Collection, France. The mutant *Anabaena* strain AMC 414 (20) was kindly provided by Dr. James Golden, University of California. *Alcaligenes hydrogenophilus* DSM 2625 was obtained from the German Collection of Microorganisms and Cell Cultures (www.dsmz.de). *Anabaena* cultures were grown under air in nitrogen free BG11 media (90) supplemented with 35 mM HEPES buffer and 10 mM sodium bicarbonate. Cultures (50 mL) were incubated in 500 mL flasks at 25°C with shaking at 160 rpm under continuous illumination of 40 μE except where specified. The medium was supplemented with spectinomycin and streptomycin [1.25 μg mL^{-1}] for growth of strain AMC 414. Ah2625 cultures were maintained on basal mineral media (12) agar plates supplemented with 10 mM sodium bicarbonate in a sealed incubator flushed with a gas mixture of 60:1.5:10 H_2:O_2:CO_2. For autotrophic growth, 20 mL liquid cultures were incubated in 160 mL sealed serum bottles flushed with a gas mixture of 60:10:10 H_2:O_2:CO_2 (unless otherwise specified) at 30°C with shaking at 160 rpm.

Co-culture and double bottle experiments were performed in 2–5 mL volumes in 14 mL sealed serum bottles and double bottles under incubation conditions described above for cyanobacteria. *Anabaena* cultures were harvested at chlorophyll *a* levels of 3–6 μg chl*a* mL^{-1} and washed twice to remove any residual antibiotic before being suspended in BG11 media. Overnight cultures of Ah2625 were grown heterotrophically in BMM media supplemented with 10 mM succinate at 30°C with shaking at 160 rpm to an OD600 of 0.8, cells were washed and suspended in the appropriate media at a dilution of 1 in 20. Cultures were stirred with a magnetic stirrer.

Knallgas bacteria isolates were obtained by spread plating serial dilutions of homogenized samples onto basal mineral media (12) agar plates supplemented with 10 mM sodium bicarbonate. The plates were incubated in a sealed incubator into which hydrogen was added to 1000-2000 ppm. Selected colonies were streaked onto R2A agar

to check for purity. To determine whether selected colonies grew autotrophically with hydrogen, isolates were tested for differential growth when restreaked onto basal mineral media plates and incubated in the sealed chamber with H_2 and air or under air without added hydrogen. Isolates showing increased growth when incubated under hydrogen were tested by GC-TCD (see below) for the ability to consume hydrogen when grown on agar slants in sealed serum bottles.

Hydrogen headspace analyses

Hydrogen content in the headspace was determined by gas chromatography (Agilent Technologies 6850 Network GC system, Agilent Technologies, U.S.A.) with a thermal conductivity detector and a HP PLOT MoleSieve 30 m x 0.53 mm x 25 µm column, a detector temperature of 250°C, an oven temperature of 50°C, and the carrier gas was argon at 3.3 ml min^{-1}. Hydrogen concentrations measured in the headspace were converted to µM hydrogen present in the liquid using Henry's gas law.

DNA and RNA purification

DNA was extracted using the Promega Wizard® Genomic DNA isolation kit, following the protocol for bacterial cells. When necessary, DNA was further purified using the Promega Wizard® SV DNA purification kit. For RNA analysis, cultures were centrifuged immediately after sampling, pellets were re-suspended in RNA later® (Ambion, USA) and stored at -20°C until extraction. RNA was extracted using the Ambion RiboPure™ RNA isolation kit (Ambion, USA). Nucleic acid integrity was analyzed using gradient gel electrophoresis for DNA, and denaturing formaldehyde gel electrophoresis for RNA. Quantification was performed by Nanodrop (Agilent). RNA was checked for DNA contamination by PCR amplification using Ah2625 specific 16S primers 988f (5′ cgaagcagagatgcwtc 3′) and 1116R (5′ gagtgcgcgttcgta 3′) (0.1 µM), and cycling conditions of 95°C 5 min, 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec, and 10 min at 72°C. When necessary residual DNA was removed using the Ambion TURBO DNA-*free*™ Kit (Ambion, USA), following the protocol for rigorous DNase treatment. RNA was reverse transcribed to cDNA using the High-

Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions.

Quantitation of gene expression

Genomic DNA from Ah2625 was extracted as described above. The 16S rRNA gene was amplified using primers 8F (5′ agagtttgatcctggctcag 3′) and 1489R (5′ taccttgttacgacttca 3′) and sequenced by Nevada Genomics Center (Reno, Nevada) (52, 98). Primers targeting *hupS* (802F 5′ accacctacaacgcctgttc 3′, 957R 5′ ttcaatgccgaactgatgaa 3′) and the 16S rRNA (988F, 1116R) genes of Ah2625 (Accession number S56898) were designed using aligned sequences of Ah2625 from the NCBI database with Primer3 software (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). Degenerate primers targeting the *rpoB* gene sequence of Ah2625 were designed using the *rpoB* gene sequences of *R. eutropha* H16 and JMP 134 (Accession numbers: 637692605 and 637692606, and 640449583 and 640449584) (1600F 5′ adcgysccgtcgcaaakcgysccgtcgcaa 3′, 1910R 5′ ttgacgcrsttgwtcagc 3′). Primers were checked for specificity *in silico* using BLAST analysis, and amplicons were sequenced to ensure specificity to target genes. No amplification of PCC 7120 DNA was detected with any Ah2625 targeted primers.

Relative expression of *hupS* was quantified using Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). PCR reactions contained 1 X master mix, either 120 nM each *hupS* primer, 300 nM each *rpoB* primer, or 300 nM 988F and 200 nM 1116R primers, and nuclease free water to a total reaction volume of 20 µL. Real time PCR was conducted with an ABI Prism 7700 sequence detection system (Applied Biosystems). Reactions were cycled through 50°C for 2 min, 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. Data collection was performed during the last step of each cycle at 72°C. RT-qPCR reactions were carried out in triplicate, a no template control was included to ensure no contamination, and a no RT control was included to ensure no DNA contamination. Standard curves were generated and included with each PCR run for reaction efficiency calculation and quantification of gene expression. PCR amplicons using *hupS*, 16S rRNA and *rpoB* primers were generated, gel purified using Qiagen QIAquick DNA clean up kit and sequenced to

ensure purity and primer specificity. Standard curves were constructed using tenfold serial dilutions over a 7 point range. Reaction efficiencies were between 90 and 110% and r^2 values > 0.99. Expression of *hupS* is shown in arbitrary units and was normalized to the expression of *rpoB* (27, 75) and 16S rRNA (21, 79) genes (1). Error bars on PCR graphs are the standard deviation from the number of replicate bottles combined with the PCR reactions (1). Error bars on all other graphs indicate range. Quantification of *hupS* copy numbers using genomic DNA template was carried out under identical PCR conditions. Standard curves were generated over a linear range of 10^7 to 10 copies, the number of gene copies in a known amount of DNA was calculated as in Ritalahti et al. (77).

Analytical methods

Chlorophyll *a* (chl*a*) concentrations were measured using methanol as the extraction solvent (56, 97). Chl*a* concentration was determined using the microplate reader method (97) with a BIO-TEK Synergy II microplate reader with a path length of 0.41 cm. Protein measurements were carried out using the BCA™ Protein Assay kit (Thermo Scientific, USA). Cell pellets were frozen at -80 °C then re-suspended in 0.1M NaOH and heated at 95 °C for 10 min before being assayed.

Membranes were prepared for MBH assay (57), without the sonication and washing steps. Membranes were suspended in 20 mM potassium phosphate buffer (pH 7.0) and homogenised by passage through 25 gauge needles. The enzyme assay was carried out using a modified method (57, 76, 80). To the main compartment of an anaerobic cuvette (Model 28UV10, Precision Cells Inc.) was added phosphate buffer (20 mM), methylene blue (0.075 mM), glucose (0.2 µmol/assay), glucose oxidase and catalase (1 U/assay) in a final volume of 3 mL. Homogenised membrane (200 – 500 µg) protein was added to the secondary compartment of the cuvette. The solution was sparged with hydrogen, evacuated and flushed with hydrogen before addition of enzyme to the bulk liquid. The reduction of methylene blue as the electron acceptor was measured over 30 min at 600 nm.

Sample collection for SIP experiments

White clover (*Trifolium repens*) and Hairy vetch (*Visia villosa*) plants on the Georgia Institute of Technology campus in Atlanta, GA were unearthed with roots intact in 5-8 cm of soil. The rhizosphere soil was collected by removing bulk soil and separating the root associated soil with a sterile spatula. Plant matter and root nodules were removed, and the rhizosphere soil was used immediately for SIP incubations.

Cyanobacteria-dominated microbial mats were collected from Elkhorn Slough, CA; Everglades National Park, FL; Sippewissett Salt Marsh, MA; Obsidian Pool, Yellowstone National Park (YNP), WY; and Sperm Pool, YNP, WY. Two additional microbial mats were collected from a lagoon spillway and a water-filled ditch near Lakeland, FL, and a cyanobacteria mat grown in an aquarium in our laboratory was used as well. After the mats were collected, they were shipped or transported on ice to the Georgia Institute of Technology and SIP incubations were prepared upon arrival. The mats from Elkhorn Slough and Sippewissett Salt Marsh were highly stratified, and only the green layer (top 1-3 mm) was used in the SIP incubations.

Stable Isotope Probing

The SIP experiments included three treatments: one with $^{13}CO_2$ and H_2 in the headspace, a control with $^{13}CO_2$ and no H_2, and a control with $^{12}CO_2$ and H_2. The treatment with $^{13}CO_2$ without H_2 controlled for ^{13}C-labeling of bacteria due to autotrophic growth with endogenous electron donors or the incorporation of inorganic carbon during heterotrophic growth (33, 41). DNA extracted from the treatment with $^{12}CO_2$ provided a control for endogenous ^{13}C and the location of ^{12}C-DNA in CsCl gradients following isopycnic ultracentrifugation. For incubations with soil, 5 g of soil were used for the experiments with 800 ppm H_2 and 2 g of soil were used for the experiments with lower H_2 concentrations. The moisture content of the white clover soil and vetch soil was 47% and 40% water holding capacity, respectively. Soil was placed at the bottom of 2.1 L flasks that were subsequently crimp-sealed with a butyl rubber stopper. The flasks were flushed for 1 minute with air that was passed through a 5 M NaOH solution to reduce ambient $^{12}CO_2$. $^{13}CO_2$ or $^{12}CO_2$ was produced by acidifying 0.1 g of $NaH^{13}CO_3$ or $NaH^{12}CO_3$ in evacuated serum bottles, and 10 ml of CO_2 was injected into each flask. The overpressure was released and H_2 was added to the desired concentration by injecting the

appropriate volume of H_2 gas. The soil was incubated in the dark, without shaking, at 22-25°C. The soil was removed and stored at -80°C at the end of the experiment.

The conditions for the SIP incubations with the microbial mat samples are listed in Table 3. When bicarbonate was used as the labeled substrate, water collected at the sampling site was acidified and put under a vacuum to remove endogenous inorganic carbon. The pH was adjusted to 7 prior to adding ^{13}C-labeled or unlabeled bicarbonate, and then the pH was returned to the value measured at the sample site. The water was filter sterilized and added to the mat sample in a serum bottle. Mat material was not incubated in liquid if incubations were prepared with $^{13}CO_2$ as the labeled substrate. At the end of the incubation, samples were removed and stored at -80°C.

Isopycnic centrifugation and gradient fractionation

DNA was extracted from frozen samples using the MoBio PowerSoil DNA extraction kit according to the manufacturer's directions. Extracted DNA (1 μg) was added to a CsCl solution in TE buffer (pH=8.0) to a final volume of 2.0 ml and an average density of 1.729 g ml^{-1}. The ultracentrifugation tubes were sealed and centrifuged in a S120-VT rotor at 190,000 g_{av} and 20°C for at least 60 hours. The CsCl gradients were fractionated from bottom to top into 150 μl fractions by displacing the gradient with sterile water from the top of the tube with a syringe pump. The density of each fraction was determined by measuring the refractive index of a subsample using an AR200 digital refractometer (Leica Microsystems). One microliter of glycogen (20 μg ml^{-1}) was added to each fraction and DNA was precipitated from the CsCl fractions by adding 2 volumes of polyethylene glycol 6000 and incubating at room temperature for 2 hours (59). After centrifuging the precipitated DNA for 20 minutes (17,200 *g*), the PEG solution was decanted and the DNA was washed twice with 70% ethanol and resuspended in 30 μl TE (pH=8.0) overnight at 4° C. DNA from gradient fractions was stored at -80°C.

Denaturing Gradient Gel Electrophoresis

Amplicons from gradient fractions were generated with primers 341f-GC and 534r as described previously (88). DGGE was performed using the DGGEEK-2001 system (C.B.S. Scientific, Solana Beach, CA). PCR products (30 μl) were loaded onto

8% (v/v) polyacrylamide gels with a 30-70% denaturant gradient, and electrophoresis was performed at 60°C for 16 hours at 70 V. Gels were imaged after staining with GelRed (Biotium, Inc., Hayward, CA) for 1 hour, and selected bands were excised and reamplified with primers 341f and 534r.

PCR, cloning, and sequencing

PCR amplification of 16S rRNA genes from gradient fractions was done using universal bacterial primers 8f and 1492r. The *hyd*B-like gene was amplified as described previously (25). PCR products were ligated into the vector pGEM T easy (Promega) by following the manufacturer's recommended protocol. Following transformation of plasmids into host cells, colonies were selected by blue/white screening. Cloned 16SrRNA genes were screened with DGGE by using the amplicons produced with vector-specific primers as the DNA template for nested PCR with the DGGE primer set 341f-GC and 534r. Selected clones were sequenced using vector-specific primers by Genewiz, Inc. (South Plainfield, NJ). Raw sequence data were assembled into full-length sequences using the program SEQMAN II (DNASTAR, Inc., Madison, WI), and analyzed using nucleotide BLAST.

Fluorescence in situ hybridization

FISH probes were designed using the Design Probe and Probe Match functions with ARB software (58). Sequences closely related to the target sequences were retrieved using BLAST searches (3), chimera checked using the program Pintail (13), and added to the ARB database. Possible probes were generated, and checked for matches in the Greengenes (29), SILVA (74), and RDP (23) databases. BLAST searches (3) were also carried out to determine if there were any sequences containing the proposed FISH probe target site that were not in the databases. Newly designed probes were validated as described by (28), using the Clone-FISH technique (71, 82).

Biomass samples were collected and fixed for FISH analysis in ethanol and paraformaldehyde for analysis of Gram positive and Gram negative cells respectively (28). Fixation was carried out as described by Daims et al. (28). FISH hybridizations were carried out as described by Amann (5). Probes used and their corresponding

formamide concentrations are listed in Table 2. Probes were labelled with either Cy3, Alexa 546, or FLUOS fluorochromes and purchased from Integrated DNA Technologies (USA). All FISH experiments included the following controls: the EUB probe (4) was applied to ensure cell permeability was adequate, and the non-EUB probe (96) used to assess the level of background auto-fluorescence in the sample after hybridization. Slides were stored at -20 °C if not immediately viewed.

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TABLES

Table 2

Oligonucleotide FISH probes used in this study. FA = formamide; n/a = not applicable.

Probe name	*Sequence (5′ - 3′)*	*Target*	*FA (%)*	*Reference*
EUB338	GCTGCCTCCCGTAGGAGT	Most Bacteria	35	(4)
Non - EUB	ACTCCTACGGGAGGCAGC	Nil	n/a	(96)
OPDG454	GAGGTATTAGCCCAGCGC	Clone OPB1	25	this study

Table 3

Incubation conditions for SIP experiments with microbial mat samples. ND = not determined, because water was not collected.

Sample	Sample Mass (g)	^{13}C label	Initial headspace conditions	pH	Temperature (°C)
Aquarium Mat	2	5 mM $NaH^{13}CO_3$	Air + 1000 ppm H2	9.8	25
Elkhorn Slough 1	2	$^{13}CO_2$	Air + 1000 ppm H2	7.1	30
Elkhorn Slough 2	1	4 mM $NaH^{13}CO_3$	Air + 1000 ppm H2	8.1	30
Everglades EC2T	1	5 mM $NaH^{13}CO_3$	87.5% N2, 10% O2, 2.5% H2	7.4	30
Everglades EC2B	1	5 mM $NaH^{13}CO_3$	92.5.5% N2, 5% O2, 2.5% H2	7.4	30
Everglades EO2	1	5 mM $NaH^{13}CO_3$	87.5% N2, 10% O2, 2.5% H2	8.2	30
Everglades TC1	1	5 mM $NaH^{13}CO_3$	87.5% N2, 10% O2, 2.5% H2	7.7	30
Spillway, FL	2	4 mM $NaH^{13}CO_3$	Air + 1000 ppm H2	6.6	30
Ditch, FL	2	4 mM $NaH^{13}CO_3$	Air + 1000 ppm H2	6.6	30
Sippewissett 1	1	4 mM $NaH^{13}CO_3$	Air + 1000 ppm H2	7.9	30
Sippewissett 2	1	4 mM $NaH^{13}CO_3$	Air + 1000 ppm H2	8.0	30
Sperm Pool 1	0.9	$^{13}CO_2$	87% N2, 13% O2, 1000 ppm H2	ND	42
Sperm Pool 2	0.9	$^{13}CO_2$	87% N2, 13% O2, 1000 ppm H2	ND	52
Obsidian Pool 1	1	10 mM $NaH^{13}CO_3$	92% N2, 3.5% O2, 4.5% H2	6.7	52
Obsidian Pool 2	1	10 mM $NaH^{13}CO_3$	87% N2, 13% O2, 1000 ppm H2	6.3	37

FIGURES

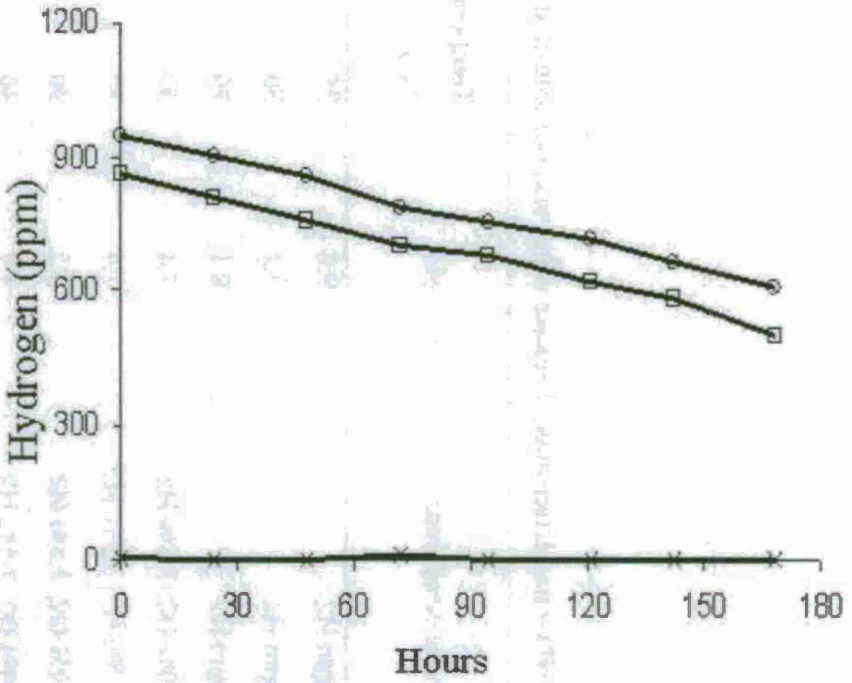

Figure 3.11. Analysis of the hydrogen concentration during incubations with a microbi mat grown in a laboratory aquarium. The data points represent the mean hydrogen concentration for duplicate measurements of $^{13}CO_2$ without H_2 (×), $^{12}CO_2$ with H_2 (○), and $^{13}CO_2$ with H_2 (□) treatments.

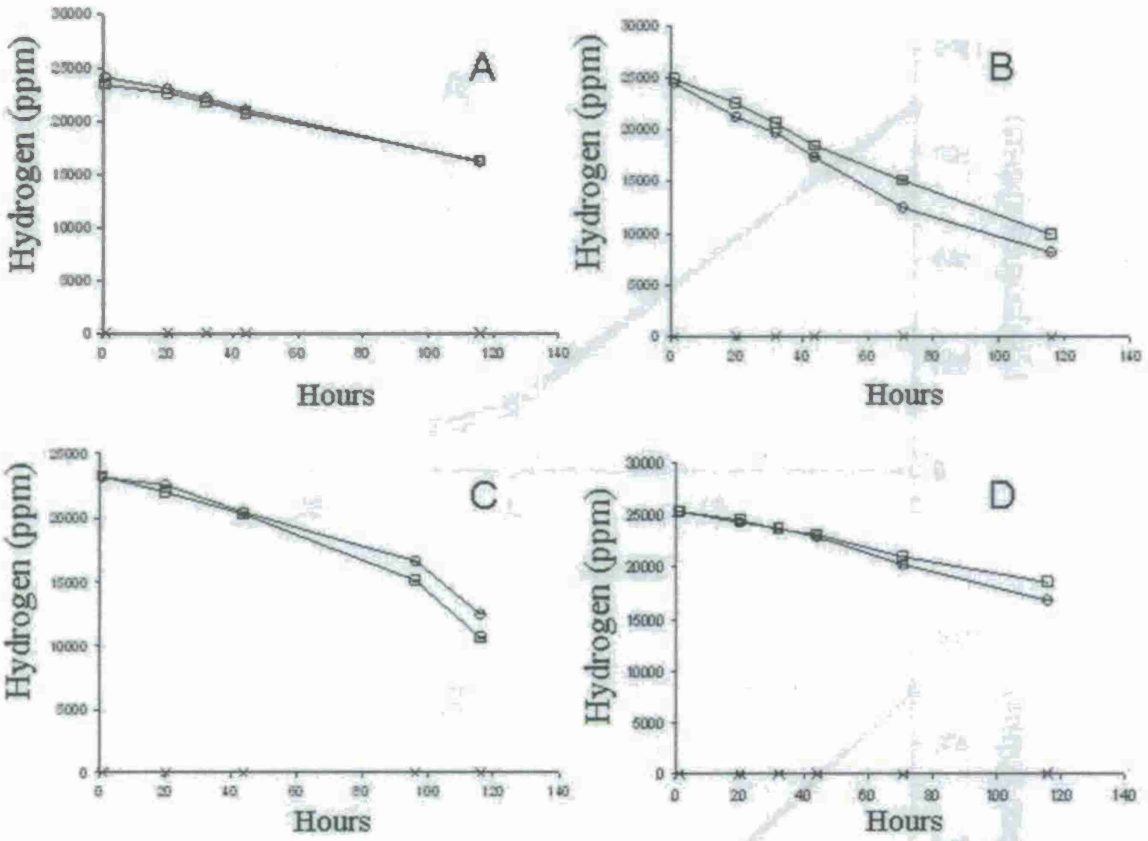

Figure 3.12. Analysis of the hydrogen concentration during incubations with microbial mats EC2T (A), EC2B (B), EO2 (C), and TC1 (D) collected from Everglades National Park. The data points represent the mean hydrogen concentration for duplicate measurements of $^{13}CO_2$ without H_2 (×), $^{12}CO_2$ with H_2 (○), and $^{13}CO_2$ with H_2 (□) treatments.

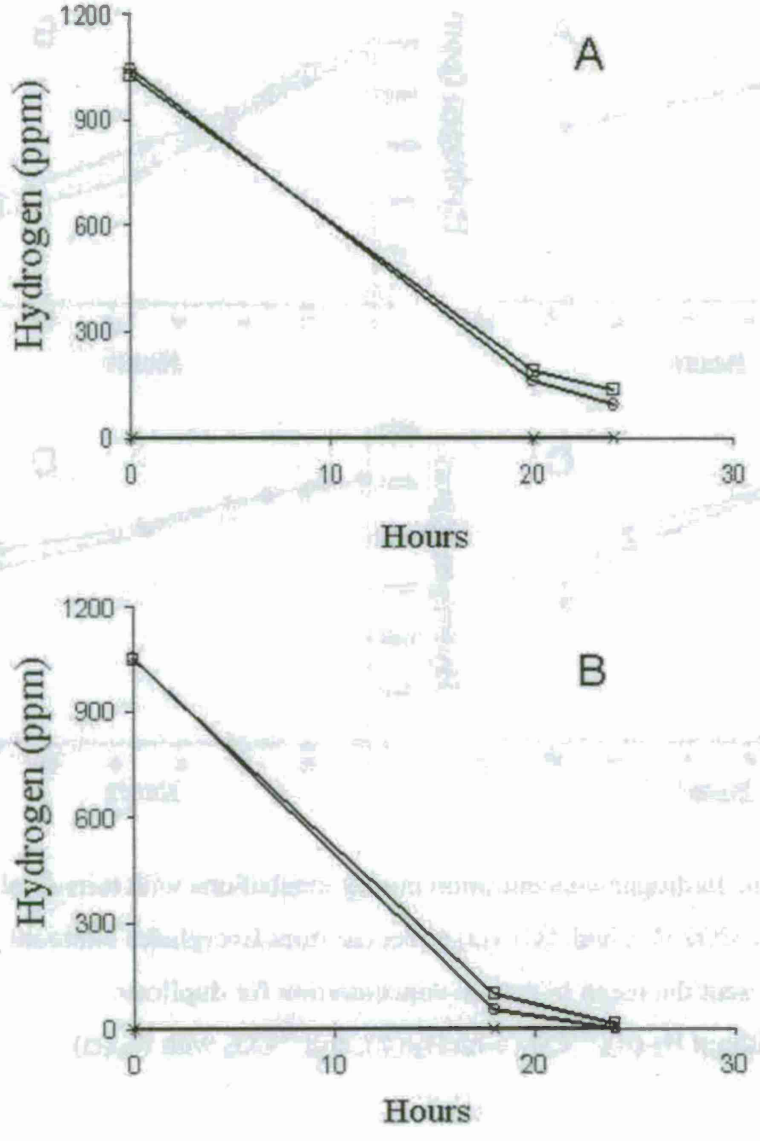

Figure 3.13. Analysis of the hydrogen concentration during incubations with microbial mats collected from a spillway (A) and ditch (B) near Lakeland, FL. The data points represent the mean hydrogen concentration for duplicate measurements of $^{13}CO_2$ without H_2 (×), $^{12}CO_2$ with H_2 (○), and $^{13}CO_2$ with H_2 (□) treatments.

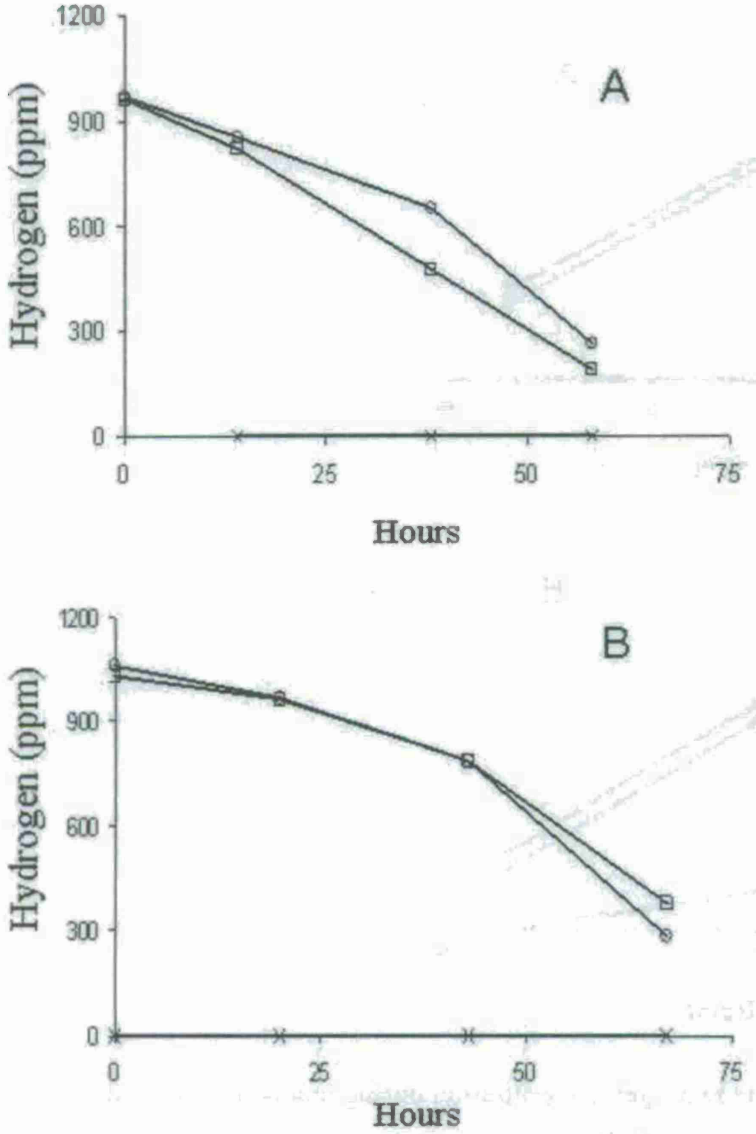

Figure 3.14. Analysis of the hydrogen concentration during incubations with the top green layer from microbial mats collected from Sippewissett Salt Marsh: Sippewissett 1 (A) and Sippewissett 2 (B). The data points represent the mean hydrogen concentration for duplicate measurements of $^{13}CO_2$ without H_2 (×), $^{12}CO_2$ with H_2 (○), and $^{13}CO_2$ with H_2 (□) treatments.

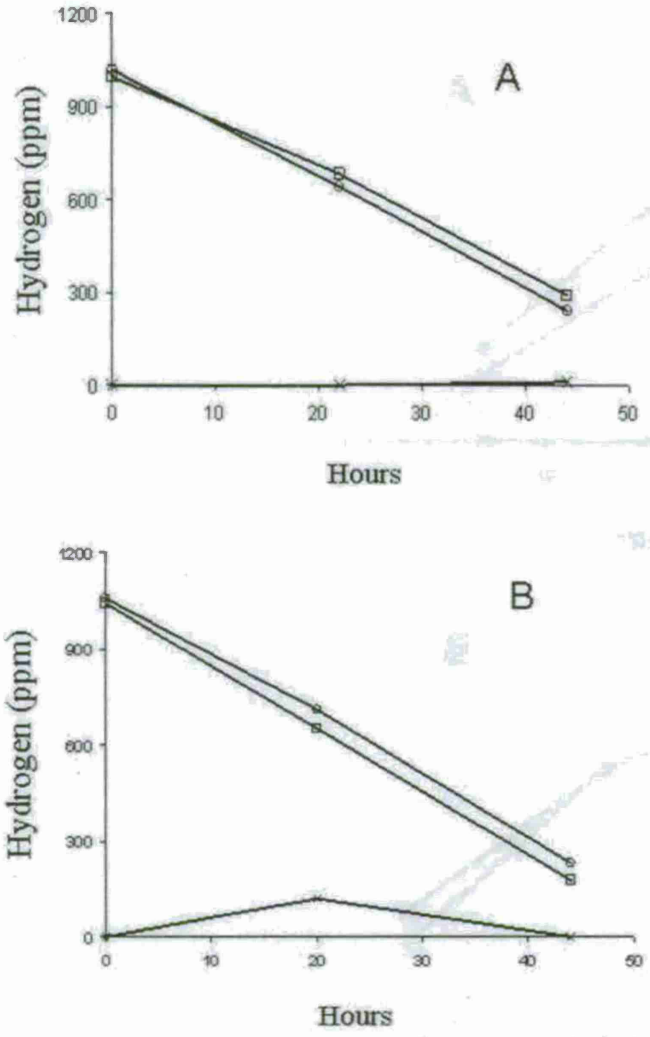

Figure 3.15. Analysis of the hydrogen concentration during incubations with the top green layer from microbial mats collected from Sperm Pool in Yellowstone National Park: Sperm Pool 1 (A) and Sperm Pool 2 (B). The data points represent the mean hydrogen concentration for duplicate measurements of $^{13}CO_2$ without H_2 (×), $^{12}CO_2$ with H_2 (○), and $^{13}CO_2$ with H_2 (□) treatments.

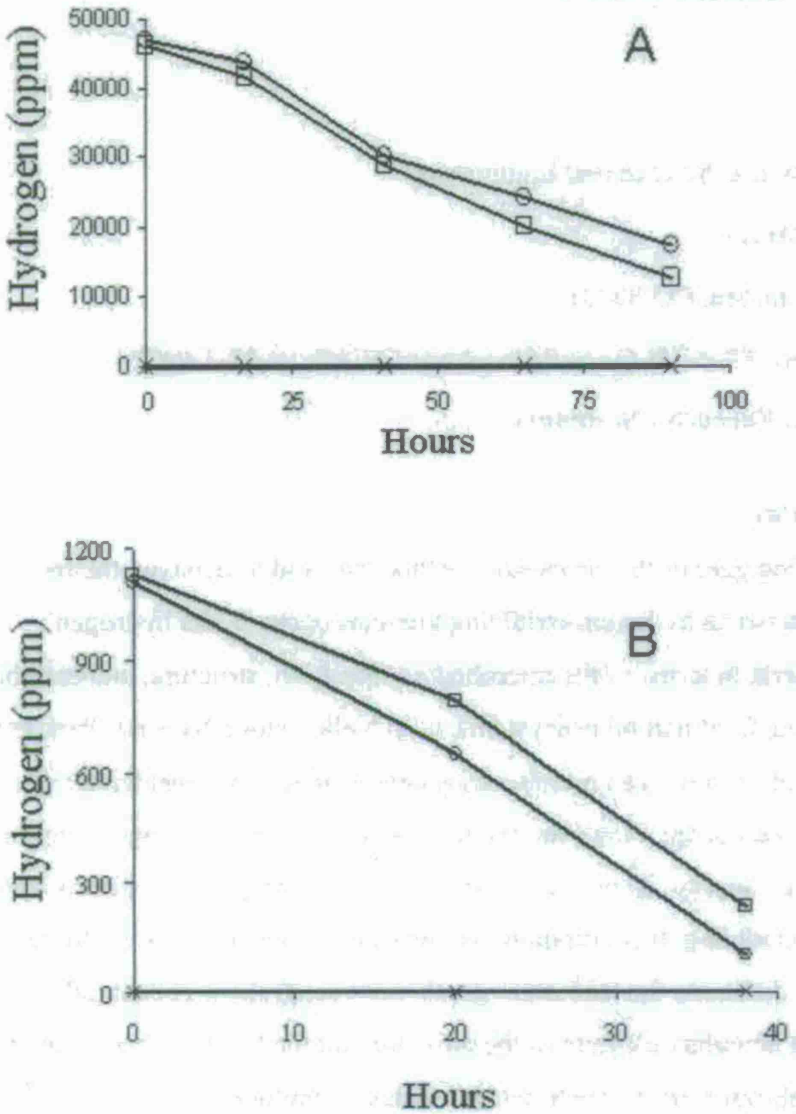

Figure 3.16. Analysis of the hydrogen concentration during incubations with the top green layer from microbial mats collected from Obsidian Pool in Yellowstone National Park: Obsidian Pool 1 (A) and Obsidian Pool 2 (B). The data points represent the mean hydrogen concentration for duplicate measurements of $^{13}CO_2$ without H_2 (×), $^{12}CO_2$ with H_2 (○), and $^{13}CO_2$ with H_2 (□) treatments.

Section 2: Molecular phylogenetic determination of knallgas:cyanobacteria interactions within natural ecosystems

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Spear, Final Report, Executive Summary

PROJECT DESCRIPTION

The overarching goal of this proposal is to describe and understand the basis for hydrogen exchange between hydrogen-oxidizing knallgas bacteria and hydrogen producing cyanobacteria in terms of the microbial composition, structure, and metabolic underpinnings in a couple of natural ecosystems, with Yellowstone National Park as the dominant site. The Yellowstone geothermal ecosystem is generally considered to be supported by sulfur metabolism. However, we have recently shown through a thorough molecular phylogenetic survey, along with *in situ* aqueous hydrogen (H_2) determination and thermodynamic modeling, that communities instead are dependant on hydrogen-metabolism and may dominate this and other geothermal ecosystems (Spear, 2005). Hydrogen is the most abundant element in the universe and the basis of diverse microbial energy metabolisms throughout the bacterial and archaeal phylogenetic domains (Stevens, 1995; White, 1988). This wide phylogenetic occurrence suggests that H_2 metabolism arose early in the evolution of life, perhaps even in the universal ancestor, the progenote (Woese, 1998). Hydrogen-metabolizing microorganisms are likely involved in several geological processes, including the formation of ore bodies and the erosion of rock in all environments.

BRIEF REVIEW OF WORK

As proposed, the work to be conducted at the Colorado School of Mines (CSM) was expected to last three years, and this report serves as a final summary of our findings.

The work involved the hiring of a graduate student, Chuck Pepe-Ranney, who was able to conduct the bulk of his Ph.D. work under this funding. Work was divided into a series of tasks the first of which involved field work in Yellowstone National Park to examine the aqueous geochemistry (hydrogen concentrations) in hotsprings along with sample collection of material for microbiological analyses. Water chemistry and microbial analyses occurred in the Spear environmental microbiology laboratory at CSM. All of our work involved a close collaboration with Jim Spain at Georgia Tech as the tasks moved forward, and members from both groups met both in the field and shared talks at conferences. The second task was also performed at CSM to amplify genes of interest out of the environmentally obtained samples and compare them to public databases. This task also involved the collaboration of both the student and the PI (Spear) with John Peters and Eric Boyd at Montana State University. Third, a couple of highly relevant tangential findings were pursued with collaborators Alex Sessions at Caltech along with William Berelson and Frank Corsetti at the University of Southern California (USC). With collaborator Sessions at Caltech, we used some stable isotope probing analysis of both *in situ* natural communities as well as cultivated representatives from those communities to consider the deuterium : hydrogen ratio (D:H ratio) in extracted lipids from Yellowstone hotsprings. With collaborators Berelson and Corsetti at USC, we were able to analyze the hydrogenase enzymes and microbial community of a novel cyanobacterially driven laminated, mineralized, microbial mat in the form of living stromatolites that grow along the rim of a Yellowstone hotspring.

STATUS OF EFFORT

As of 31 March 2011, the work for this AFOSR funded project is now complete. Funds for this project arrived at the Colorado School of Mines on 9 September 2008. A Ph.D. graduate student in the Division of Environmental Science and Engineering at CSM, Charles (Chuck) Pepe-Ranney was hired to carry out the proposed work. He is expected to complete his thesis and graduate in the Spring of 2012. Two field trips in 2008 were conducted to Yellowstone National Park to collect biomass samples for the proposed work under the National Park Service authorized research permit to Co-PI John R. Spear. Four field trips were conducted in 2009 including one trip with Co-Pi Spain in

June, and one trip with collaborator Alex Sessions (Caltech) and graduate Student (Maggie Osburn, Caltech). Samples were collected in duplicate for immediate shipment to Co-PI Jim Spain at Georgia Tech, and for laboratory work at the Colorado School of Mines (CSM). Four field trips were completed in 2010 in support of sample acquisition for laboratory analyses; one of those trips with collaborator Spain. Preliminary sample analyses (e.g. microscopy, scanning electron microscopy, and molecular approaches—16S rRNA and functional gene analysis) were completed at CSM. Chuck Pepe-Ranney extracted DNA and standardized their concentrations from 20 hotspring samples to run on a bar-coded, multiplexed, Roche/Life Sciences 454 Pyrosequencer at the University of Colorado Health Science Center in February, 2010.

The ubiquity of the metabolism of hydrogen oxidation in the Tree of Life makes it prohibitively difficult to target hydrogen oxidizers (i.e. knallgas microbes) via the 16S rRNA gene. Instead, we have thoroughly investigated the phylogeny of the large subunit of the NiFe hydrogenase enzyme searching for possible functional gene primer sites (going after the gene instead of the microbe). The known NiFe hydrogenases are split into 4 major groups phylogenetically. One group in particular is comprised of non-cyanobacterial "uptake" hydrogenase genes. Due to the remarkably high genetic diversity of the NiFe hydrogenase, it is impossible to design primers that will amplify all known NiFe's *in silico*. The diversity, however, should allow us to target the non-cyanobacterial uptake group to the exclusion of the other phylogenetic groupings that would not be associated with the knallgas metabolism. We have yet to find an appropriate primer pair for all the aforementioned uptake genes but each of the major subgroups within the uptake-clade can be targeted by a specific primer pair. We hoped to establish a manageable set of primer combinations that encompass the entire uptake group and then investigate the diversity of such genes within our samples (above) via PCR and sequencing (culture-independent methods). This work remains on-going at the completion of this grant.

In May of 2009, the Co-PIs and Chuck Pepe-Ranney had a meeting at the American Society for Microbiology Annual Meeting in Philadelphia, PA to discuss the Work Plan and goals accomplished to date and what needs to be done in the future. In June 2009, Co-Pi Spear took collaborators Alex Sessions and Maggie Osburn (Caltech)

to 20 Yellowstone hotsprings for sample collection to measure the ratio of deuterium to hydrogen in the lipid biomass of microbiota. Such analysis is novel and important for the project to better ascertain the kinds of microbes involved in knallgas and hydrogen metabolism. In August 2009, Co-PI Spear and Chuck Pepe-Ranney conducted dissolved aqueous phase hydrogen measurements in 20 hotsprings associated with the project. Additional collaborations have been initiated with John Peters' group at Montana State University, Alex Sessions' group at Caltech and involvement with the Colorado Sequencing Consortium at the University of Colorado Health Science Center in support of this project. Several trips were made to the hotspring with the living stromatolites along its edge, a few samples collected, with subsequent microbiological analysis at CSM and geological / geochemical analysis at USC by collaborators Corsetti and Berelson, respectively.

ACCOMPLISHMENTS

- Ph.D. graduate student Chuck Pepe-Ranney hired, and nearly finished.
- Two field trips conducted in 2008.
- Four field trips conducted in 2009.
- Co-PI Spain on one field trip in June of 2009.
- Four field trips conducted in 2010.
- Co-Pi Spain on one field trip in August of 2010.
- Meeting of Co-PIs at the 2009 and 2011 American Society for Microbiology Annual Meetings.
- Collaborator Alex Sessions (Caltech) on one field trip in June of 2009.
- Initial sample analysis—completed.
- Collaborative contacts at three locations—established.
- Data Collection for hydrogen concentration and geochemistry completed.
- Hydrogen Concentrations at 20 hotsprings conducted; 4 – 100 nM concentrations.
- 7 Peer-Reviewed publications either in print, accepted or submitted.

PERSONNEL SUPPORTED

Ph.D. Graduate Student Charles (Chuck) Pepe-Ranney hired. Currently enrolled as an Environmental Science and Engineering graduate student at CSM. Expected graduation, Spring, 2012. Support role provided by Shannon Ulrich, Ph.D. graduate student in Environmental Science and Engineering at CSM.

PUBLICATIONS

Boyd, E.S., T.L. Hamilton, J.R. Spear, M. Lavin and J.W. Peters. 2010. [Fe-Fe]-hydrogenase in Yellowstone National Park: Evidence for dispersal limitation and phylogenetic niche conservatism. ISME J. 4:1485-1495.

Berelson, W.M., F.A. Corsetti, W. Beaumont, D.E. Hammond, C. Pepe-Ranney, and J.R. Spear. 2011. Hot spring siliceous stromatolites from Yellowstone National Park: assessing growth rate and laminae formation. Geobiology. 9:411-424.

Osburn, M.R., A.L.Sessions, C. Pepe-Ranney and J.R. Spear. 2011. Hydrogen-isotopic variability in fatty acids from Yellowstone National Park hot spring microbial communities. Geochim. Cosmochim. Acta. 75:4830-4845.

Pepe-Ranney, C., W.M. Berelson, F.A. Corsetti, M. Treants and J.R. Spear. 2011. Cyanobacterial construction of hot spring siliceous stromatolites in Yellowstone National Park. Environ. Microbiol. In Press.

Ross, K.A., L.M. Feazel, C.E. Robertson, B.Z. Fathepure, K.E. Wright, R.M. Turk, M.M. Chan, N.L. Held, J.R. Spear, and N.R. Pace. Phototrophic phylotypes dominate microbial mats of hotspring mesothermic zones in Yellowstone National Park. Submitted to PLoS ONE.

Mata, S.A., C.L. Harwood, F.A. Corsetti, N.J. Stork, K. Eilers, C. Pepe-Ranney, W.M. Berelson and J.R. Spear. Influences of gas production and filament orientation on stromatolite microfabric. Submitted to Palaios.

INTERACTIONS / TRANSITIONS

- An organizational conference call with Co-PIs in 2008
- Co-PI Meeting at the American Society for Microbiology Annual Meeting, 2010

- Collaborative discussions with 3 institutions for supporting work (MSU, Caltech and UCHSC)
- Co-PI Spear and grad student Pepe-Ranney attended the Metagenomics 2008 meeting at UC San Diego in November, 2008, to learn tools, techniques and approaches for analyzing, organizing and working with large pyrosequencing generated data sets that will result from application to this project.
- Co-PI Spear and grad student Pepe-Ranney attended the Joint Genome Institute Users Meeting, March, 2009, to further learn tools, techniques and approaches for analyzing, organizing and working with large pyrosequencing generated data sets that will result from application to this project.

CONSULTATIVE, TECHNOLOGY ASSISTS, NEW DISCOVERIES, HONORS AND AWARDS

Other than all of the new work presented in the above listed seven publications, no consultative, technology assists, honors or awards to report. However, it is quite likely that our reportings will lead to significant advancements in the better understanding of relations between microbes in community. That information will likely have benefits for the manipulation and exploitation of natural processes for things like biofuel development.

ABSTRACTS AND SIGNIFICANT FIGURES FROM PUBLISHED, ACCEPTED AND SUBMITTED WORKS

Boyd, E.S., T.L. Hamilton, J.R. Spear, M. Lavin and J.W. Peters. 2010. [Fe-Fe]-hydrogenase in Yellowstone National Park: evidence for dispersal limitation and phylogenetic niche conservatism. ISME J. 4:1485-1495.

ABSTRACT

Hydrogen (H_2) has an important role in the anaerobic degradation of organic carbon and is the basis for many syntrophic interactions that commonly occur in microbial communities. Little is known, however, with regard to the biotic and/or abiotic factors that control the distribution and phylogenetic diversity of organisms which produce H_2 in

microbial communities. In this study, we examined the [Fe-Fe]-hydrogenase gene (hydA) as a proxy for fermentative bacterial H_2 production along physical and chemical gradients in various geothermal springs in Yellowstone National Park (YNP), WY, USA. The distribution of hydA in YNP geothermal springs was constrained by pH to environments co-inhabited by oxygenic phototrophs and to environments predicted to have low inputs of abiotic H_2. The individual HydA asssemblages from YNP springs were more closely related when compared with randomly assembled communities, which suggests ecological filtering. Model selection approaches revealed that geographic distance was the best explanatory variable to predict the phylogenetic relatedness of HydA communities. This evinces the dispersal limitation imposed by the geothermal spring environment on HydA phylogenetic diversity even at small spatial scales. pH differences between sites is the second highest ranked explanatory variable of HydA phylogenetic relatedness, which suggests that the ecology related to pH imposes strong phylogenetic niche conservatism. Collectively, these results indicate that pH has imposed strong niche conservatism on fermentative bacteria and that, within a narrow pH realm, YNP springs are dispersal limited with respect to fermentative bacterial communities.

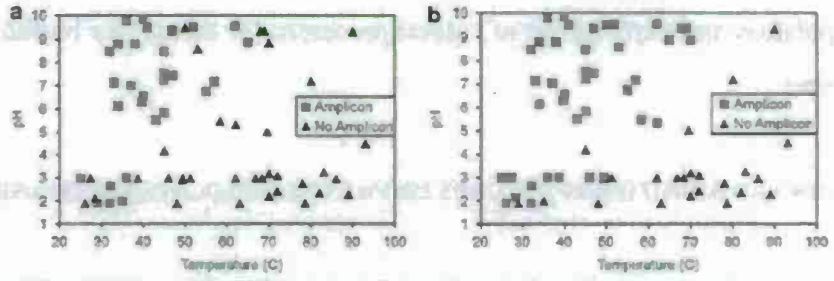


Figure 1 Distribution of *hydA* (**a**) and *bchL* (**b**) in sediment and mat sampled from 65 geothermal springs from four geographic locations in Yellowstone National Park as a function of spring water pH and temperature. Red squares denote environments where amplicons were detected and blue triangles denote environments where amplicons were not detected.

Berelson, W.M., F.A. Corsetti, W. Beaumont, D.E. Hammond, C. Pepe-Ranney, and J.R. Spear. 2011. Hot spring siliceous stromatolites from Yellowstone National Park: assessing growth rate and laminae formation. Geobiology. 9:411-424.

ABSTRACT

Stromatolites are commonly interpreted as evidence of ancient microbial life, yet stromatolite morphogenesis is poorly understood. We applied radiometric tracer and dating techniques, molecular analyses and growth experiments to investigate siliceous stromatolite morphogenesis in Obsidian Pool Prime (OPP), a hot spring in Yellowstone National Park. We examine rates of stromatolite growth and the environmental and/or biologic conditions that affect lamination formation and preservation, both difficult features to constrain in ancient examples. The "main body" of the stromatolite is composed of finely laminated, porous, light-dark couplets of erect (surface normal) and reclining (surface parallel) silicified filamentous bacteria, interrupted by a less-distinct, well-cemented "drape" lamination. Results from dating studies indicate a growth rate of 1—5 cm/year, however growth is punctuated. ^{14}C as a tracer demonstrates that stromatolite cyanobacterial communities fix CO_2 derived from two sources, vent water (radiocarbon dead) and the atmosphere (modern ^{14}C). The drape facies contained a greater proportion of atmospheric CO_2 and more robust silica cementation (vs. the main body facies), which we interpret as formation when spring level was lower. Systematic changes in lamination style are likely related to environmental forcing and larger scale features (tectonic, climatic). Although the OPP stromatolites are composed of silica and most ancient forms are carbonate, their fine lamination texture requires early lithification. Without early lithification, whether silica or carbonate, it is unlikely that a finely laminated structure representing an ancient microbial mat would be preserved. In OPP, lithification on the nearly diurnal time scale is likely related to temperature control on silica solubility.

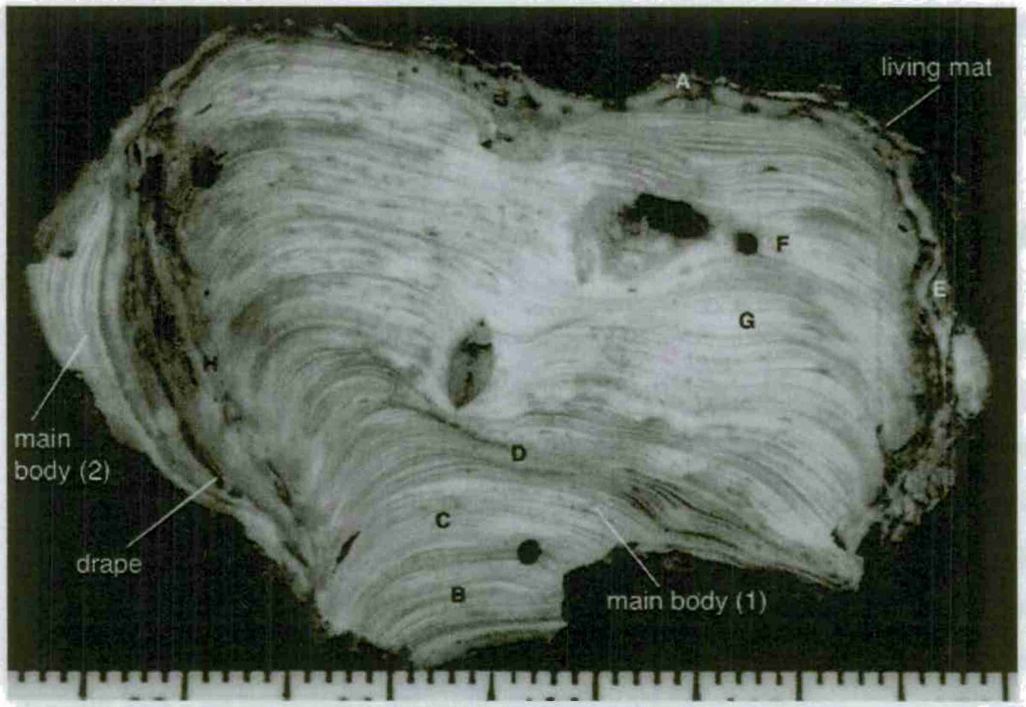


Fig. 2: Siliceous stromatolite obtained from Obsidian Prime hot spring, YNP.

Scale units at bottom of frame are 1 mm. Letters indicate sites where C, N and S measurements were made (see Table 1). Note two distict styles of lamination: "main body" and "drape". Following the growth of main body (1), growth was replaced by the drape fabric; growth returned to the main body style of lamination (main body 2). When collected, a living mat—an incipient drape—topped the structure.

Osburn, M.R., A.L. Sessions, C. Pepe-Ranney and J.R. Spear. 2011. Hydrogen-isotopic variability in fatty acids from Yellowstone National Park hot spring microbial communities. Geochim. Cosmochim. Acta. 75:4830-4845.

ABSTRACT

We report the abundances and hydrogen-isotopic compositions (D/H ratios) of fatty acids extracted from hot-spring microbial mats in Yellowstone National Park. The terrestrial hydrothermal environment provides a useful system for studying D/H fractionations because the numerous microbial communities in and around the springs are visually distinct, separable, and less complex than those in many other aquatic environments. D/H fractionations between lipids and water ranged from -374‰ to +41‰ and showed systematic variations between different types of microbial communities. Lipids produced by chemoautotrophic hyperthermophilic bacteria, such as icosenoic acid (20:1), generally exhibited the largest and most variable fractionations from water (-374‰ to -165‰). This was in contrast to lipids characteristic of heterotrophs, such as branched, odd chain-length fatty acids, which had the smallest fractionations (-163‰ to +41‰). Mats dominated by photoautotrophs exhibited intermediate fractionations similar in magnitude to those expressed by higher plants. These data support the hypothesis that variations in lipid D/H are strongly influenced by central metabolic pathways. Shifts in the isotopic compositions of individual fatty acids across known ecological boundaries show that the isotopic signature of specific metabolisms can be recognized in modern environmental samples, and potentially recorded in ancient ones. Considering all sampled springs, the total range in D/H ratios is similar to that observed in marine sediments, suggesting that the trends observed here are not exclusive to the hydrothermal environment.

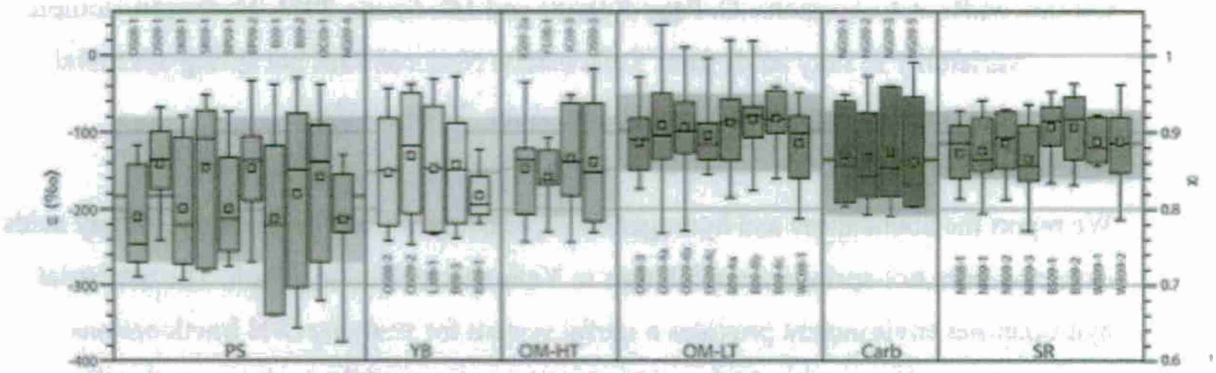


Figure 5: The range of isotopic fractionations in each sample (individual bars) and for mat types (separate colors). For each sample the boxes contain 50% of the data with the upper and lower quartile shown by the whiskers. The mean and median of the data points are indicated by the square and line, respectively. The mean values ($\pm 2\sigma$) for each group are shown in the background lines and shading.

Rhodes, M.E., J.R. Spear, A. Oren and C.H. House. 2011. Differences in lateral gene transfer in hypersaline versus thermal environments. BMC Evol. Biol. 11:199. doi:10.1186/1471-2148-11-199.

ABSTRACT

Background: The role of lateral gene transfer (LGT) in the evolution of microorganisms is only beginning to be understood. While most LGT events occur between closely related individuals, inter-phylum and inter-domain LGT events are not uncommon. These distant transfer events offer potentially greater fitness advantages and it is for this reason that these "long distance" LGT events may have significantly impacted the evolution of microbes. The most likely mechanism driving distant LGT events is microbial transformation. Theoretically, transformative events can occur between any two species provided that the DNA of one enters the habitat of the other. Two categories of microorganisms that are well known for LGT are the thermophiles and halophiles.

Results: We identified potential inter-class LGT events in a thermophilic class of Archaea, the Thermoprotei, and a halophilic class of Archaea, the Halobacteria. We then categorized these LGT genes as originating in thermophiles and halophiles respectively. While more than 68% of transfer events into Thermoprotei taxa originated in other thermophiles, less than 11% of transfer events into Halobacteria taxa originated in other halophiles.

Conclusions: Our results suggest that there is a fundamental difference between LGT in thermophiles and halophiles. We theorize that the difference lies in the different natures of the environments. While DNA degrades rapidly in thermal environments due to temperature-driven denaturization, hypersaline environments are adept at preserving DNA. Furthermore, most hypersaline environments, as topographical minima, are natural collectors of cellular debris. Thus halophiles would in theory be exposed to a greater diversity and quantity of extracellular DNA than thermophiles.

Halobacteria										
Genera	GC	CG Di's	GA/TC Di's	AC/GT Di's	Cysteine	Leucine	Threonine	Valine	Arginine	AB Ratio
Halalkalicoccus	3.80	0.70	0.60	-0.11	2.16	2.03	-2.04	3.91	-5.24	-0.10
Haloarcula	4.06	0.69	0.22	0.31	-0.99	-1.09	0.35	3.58	-2.05	0.11
Halobacterium	3.51	0.70	-0.38	0.07	-16.45	-3.59	0.64	0.18	-5.84	0.02
Haloferax	4.04	1.03	0.69	0.49	6.03	5.87	1.67	3.62	-3.53	-0.11
Halomicrobium	3.96	0.62	0.73	-0.05	2.80	0.42	3.17	3.05	-4.84	-0.06
Haloquadratum	1.00	0.21	-0.25	0.39	4.12	-1.67	-0.87	-0.78	0.70	0.01
Halorhabdus	0.86	0.43	0.49	0.03	1.17	1.54	2.56	-0.64	2.17	-0.19
Halorubrum	7.50	1.85	0.97	0.59	7.17	8.51	3.05	7.74	-4.85	-0.04
Haloterrigena	2.08	0.33	0.74	-0.44	3.61	1.00	-1.08	6.46	-4.31	0.10
Natrialba	1.82	0.51	0.37	0.16	2.80	5.00	0.87	2.72	-5.72	0.09
Natronomonas	4.05	0.99	0.16	0.54	4.88	2.30	4.14	7.84	-1.96	0.03
Thermoprotei										
Genera	GC	CG Di's	GA/TC Di's	AC/GT Di's	Cysteine	Leucine	Threonine	Valine	Arginine	AB Ratio
Acidilobus	-1.30	-0.11	0.26	-0.58	0.64	-2.21	-3.61	0.37	1.89	0.02
Aeropyrum	3.67	-0.41	0.66	0.03	-6.46	3.65	-0.52	1.91	-2.98	-0.01
Caldivirga	0.10	-0.01	-0.31	-0.02	-5.14	0.45	0.22	-0.04	-0.04	-0.05
Desulfurococcus	-2.92	-0.26	0.17	-0.53	9.87	-1.27	-1.54	-2.69	2.00	0.05
Hyperthermus	-1.69	0.11	0.01	-0.21	7.53	-1.90	-3.22	0.07	0.53	0.01
Ignicoccus	-0.24	-0.14	0.34	-0.17	-0.16	1.16	-0.40	0.76	-2.70	0.04
Ignisphaera	-1.54	0.05	0.13	-0.33	6.12	-0.96	-0.68	-1.03	0.77	-0.07
Metallosphaera	-1.79	0.18	0.05	-0.03	-0.30	-2.78	-0.85	1.34	0.45	-0.02
Pyrobaculum	2.02	-0.05	0.50	0.04	-0.14	2.58	2.50	0.46	-0.09	0.04
Staphylothermus	-0.15	0.12	0.30	-0.14	-2.67	-0.35	0.12	0.41	0.63	0.03
Sulfolobus	-1.17	0.04	0.11	-0.70	1.58	-1.09	-0.85	-1.24	0.20	0.02
Thermofilum	0.17	-0.03	0.24	-0.27	-0.26	1.34	-0.68	0.85	1.26	0.05
Thermoproteus	1.59	-0.44	0.65	0.55	8.37	-0.85	2.13	3.09	-3.53	0.01

Table 2- **Genomic halophilicity indicators:**

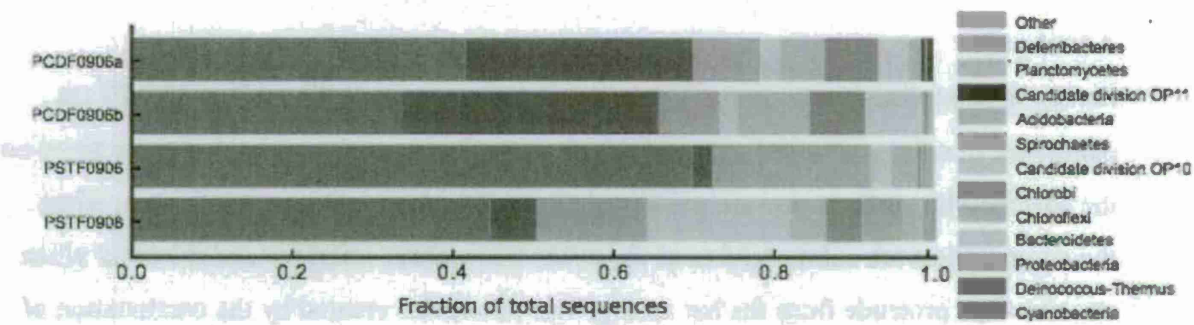
Percent differences for various indicators of halophilicity between LGT genes and non-LGT genes for the (a) Halobacteria and (b) Thermoprotei. Values for which the LGT genes are less than the non-LGT genes are shaded in green. Values for which the LGT genes are greater than the non-LGT genes are shaded in red. All values that exhibited greater than 95% confidence are shown larger and in bold font. The indicators are: GC - GC content of the gene pools. CG Di's, GA/TC Di's, AC/GT Di's– Difference in preference for 'CG', 'GA/TC', and 'AC/GT' dinucleotides given nucleotide frequencies. Arginine (CGA and CGG), Cysteine (UGU), Leucine (CUC), Threonine (ACG), and Valine (GUC) – Difference in preference for respective codons given nucleotide abundances. AA Bias – Ratio of aspartic acid and glutamic acid to arginine, lysine, and histidine.

Pepe-Ranney, C., W.M. Berelson, F.A. Corsetti, M. Treants and J.R. Spear. 2011. "Cyanobacterial Construction of Hot Spring Siliceous Stromatolites in Yellowstone National Park." Accepted to *Environmental Microbiology.*

ABSTRACT

Living stromatolites growing in a hot spring in Yellowstone National Park (YNP) are composed of silica-encrusted cyanobacterial mats. Two cyanobacterial mat types grow on the stromatolite surfaces and are preserved as two lithofacies. One mat is present when the stromatolites are submerged or at the water-atmosphere interface and the other when stromatolites protrude from the hot spring. The lithofacies created by the encrustation of submerged mats constitutes the bulk of the stromatolites, is comprised of silica-encrusted filaments and is distinctly laminated. To better understand the cyanobacterial membership and structure differences between the mats, we collected mat samples from each type. Molecular methods revealed that submerged mat cyanobacteria were predominantly one novel phylotype while the exposed mats were predominantly heterocystous phylotypes (*Chlorogloeopsis* and *Fischerella*). The novel cyanobacterium is a non-heterocystous filament and has also been found in association with travertine stromatolites in a Southwest Japan hot spring. Cyanobacterial membership profiles indicate that the heterocystous phylotypes are "rare biosphere" members of the submerged mats. The heterocystous phylotypes likely emerge when the water level of the hot spring drops. Spatial strategies for separating nitrogen fixation with oxygenic photosynthesis (i.e., heterocysts) may preclude the heterocystous cyanobacteria from dominating the submerged mats where diffusion is slower and oxygen can potentially accumulate to supersaturated levels with respect to the atmosphere. Additionally, motility in response to sedimentation, and temperature niches may play roles in surface community structuring. These living stromatolites may represent the current best analog to those that pepper the rock record.

Figure 2. The bar chart depicts the distribution of pyrosequencing reads into phyla for each pyrosequence library. Taxonomic classifications were obtained by recruiting reads to annotated reference sequences in the Silva SSURef102_NR database. Cyanobacteria is the most abundant phylum in each library. Due to the relatively large cell volume of cyanobacteria, the community biomass attributable to cyanobacteria is likely even higher than fraction of pyrosequences.



Ross, K.A., L.M. Feazel, C.E. Robertson, B.Z. Fathepoure, K.E. Wright, R.M. Turk, M.M. Chan, N.L. Held, J.R. Spear, and N.R. Pace. Phototrophic phylotypes dominate microbial mats of hots pring mesothermic zones in Yellowstone National Park. Submitted to PLoS ONE.

ABSTRACT

Five stratified, mesothermic microbial mats from Yellowstone were split into layers to examine and compare microbial diversity. Genomic DNA was extracted and ribosomal-RNA genes were amplified with universal primers. The mat assemblages were dominated by sequences representative of phototrophic bacteria, especially Cyanobacteria in top layers, and Chloroflexi in deeper layers.

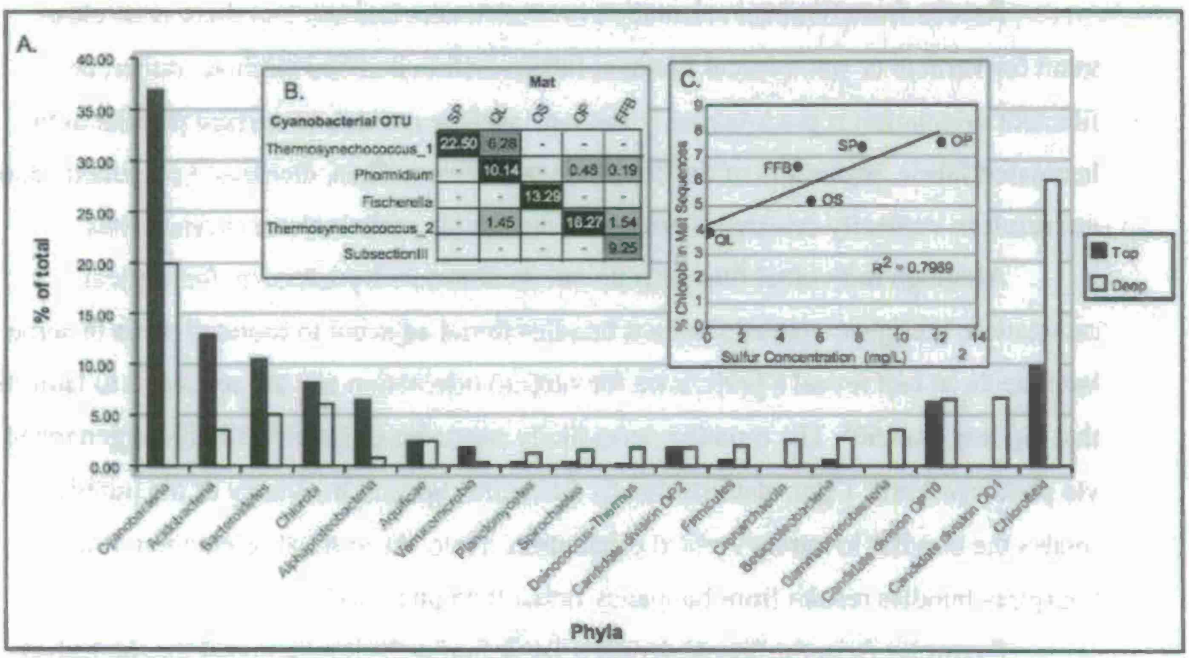


Figure 1. **(A)** Phylum-level differences observed in the top layer of mats compared to deeper layers (layers 2 and deeper). **(B)** A unique Cyanobacterial clade (99% OTU) is observed to be dominant in each mat. **(C)** Percentage of sequences per mat that are closely related to sulfur-reducing chlorobi is positively correlated with sulfur concentration of water from the springs.

Mata, S.A., C.L. Harwood, F.A. Corsetti, N.J. Stork, K. Eilers, C. Pepe-Ranney, W.M. Berelson and J.R. Spear. Influences of gas production and filament orientation on stromatolite microfabric. Submitted to Palaios.

ABSTRACT

Stromatolite microfabrics have been attributed to a phototactic response of the microbial communities that built them, where microbial filaments orient vertically with incident light during the day and lie flat at night. Here, we examine the orientation of cyanobacterial filaments within a finely laminated stromatolite from a Yellowstone National Park hot spring to test whether there is a preference for sub-vertical filaments as would be predicted for a phototactic response, or if the filaments show a different pattern.

Results from filament orientation measurements indicate that there is no clear trend for vertical or sub-vertical filament fabrics within inclined laminae; rather, the filament orientation is predominantly random, surface normal, or surface parallel to the laminated fabric, regardless of the dip of the lamination. Thus, diel-based phototaxis does not result in vertically oriented filaments at the lamina scale in these stromatolites.

Although individual filaments do not demonstrate a preference for vertical orientation, hourglass-shaped filament bundles found adjacent to rounded pores in some laminae do in fact reveal a preference for vertical orientation and are restricted to laminae that dip less than 60°. The rounded pores likely represent oxygen-rich bubbles generated via photosynthesis. Upon stabilization by filaments, upward buoyancy of the bubbles rotates the bundles toward a vertical orientation. Thus, the vertical orientation of the hourglass bundles results from buoyancy rather than phototaxis.

Examples from the Neoproterozoic Beck Spring Dolomite reveal that hourglass structures are present in the rock record and may be preferentially preserved versus the individual filaments that likely once comprised them. The presence of rounded pores (fenestrae) and hourglass structures in ancient microbialites, here termed the hourglass-associated fenestral fabric, can serve as a good indication of biogenic influences on stromatolite formation, especially in the absence of preserved filaments, and may be an indication of oxygenic photosynthesis.

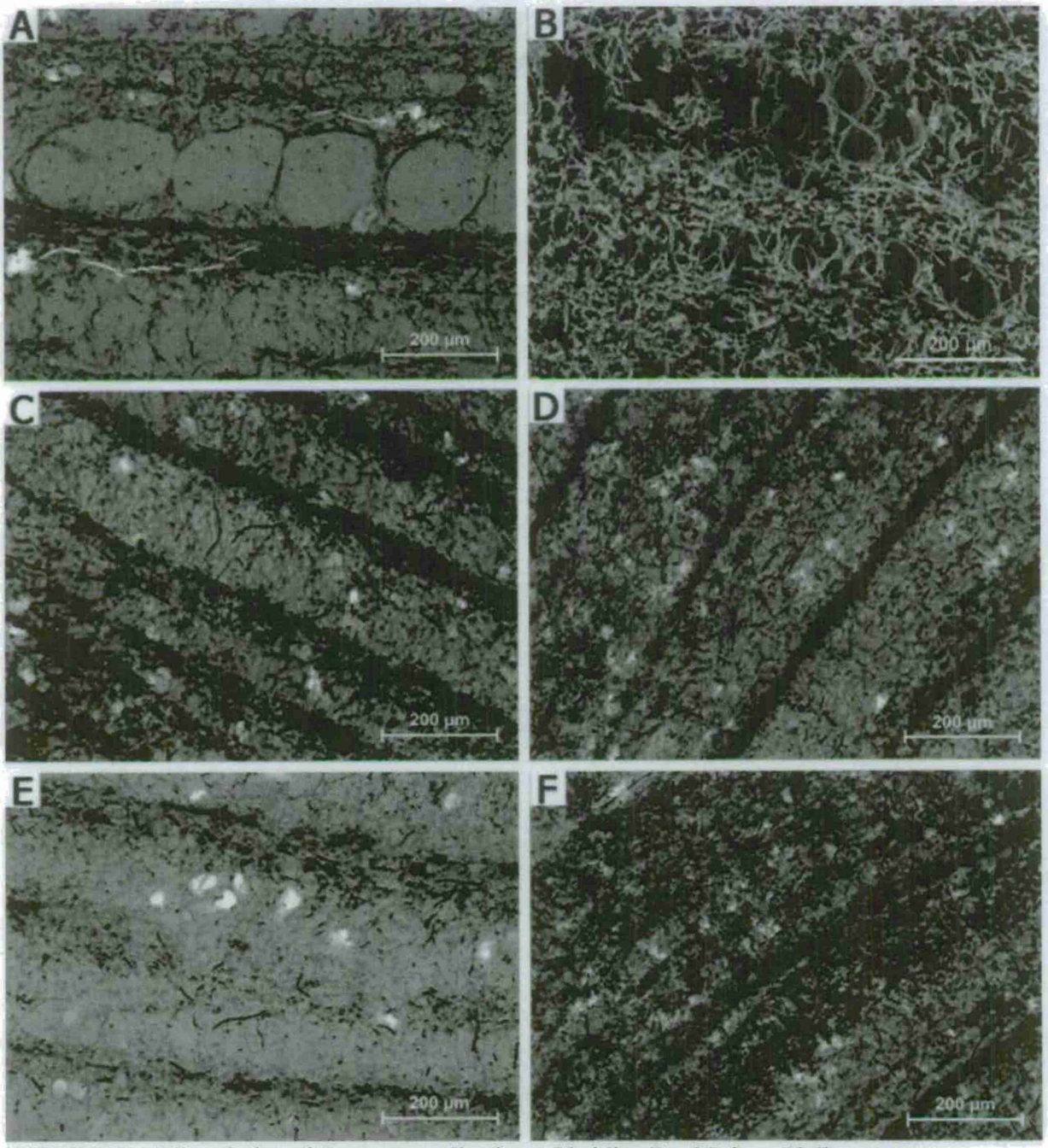


FIGURE 3—Microfacies of the stromatolite from Obsidian Pool Prime, Yellowstone National Park, USA. A) The rounded pore microfacies consists of an open filament network that surrounds rounded pores. Between adjacent pores are found hourglass-shaped filament bundles. B) Scanning electron micrograph of the rounded pore microfacies showing that the rounded pore spaces are three-dimensional spherical features that filaments contour around. C) The grading laminae microfacies is defined by

the gradual upward increase in filament density within each laminae. D) The non-grading laminae microfacies shows no distinct changes in filament density within the light laminae consisting of an open filament network. E) The ultraporous laminae microfacies is comprised of a poorly silicified open filament network in which dark laminae are weakly defined by faint increases in filament density. F) The dense laminae microfacies generally lacks an open filament network and consists primarily of densely-packed filaments alternating with silica-encrusted horizons.